Molecular and functional aspects of antimalarial drug resistance in isolates from Africa and Asia

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- **Tacoli C***,* Gai PP, Siegert K, Wedam J, Kulkarni SS, Rasalkar R, Boloor A, Jain A, Mahabala C, Baliga S, Shenoy D, Gai P, Devi R & Mockenhaupt FP (2019) Characterization of *Plasmodium vivax pvmdr1* polymorphisms in isolates from Mangaluru, India. *American Journal of Tropical Medicine & Hygiene* 2019, Jun 17. Doi: 10.4269/ajtmh.19-0224. *[Epub ahead of print]*.

- **Tacoli C**, Gai PP, Bayingana C, Sifft K, Geus D, Ndoli J, Sendegeya A, Gahutu JB & Mockenhaupt FP (2016) Artemisinin Resistance-Associated *K13* Polymorphisms of *Plasmodium falciparum* in Southern Rwanda, 2010-2015. *American Journal of Tropical Medicine & Hygiene* **95**:1090-93. Doi: 10.4269/ajtmh.16-0483.

- Wedam J, **Tacoli C**, Gai PP, Siegert K, Kulkarni SS, Rasalkar R, Boloor A, Jain A, Mahabala C, Baliga S, Shenoy D, Devi R, Gai P & Mockenhaupt FP (2018) Molecular Evidence for *Plasmodium falciparum* Resistance to Sulfadoxine-Pyrimethamine but Absence of *K13* Mutations in Mangaluru, South-western India. *American Journal of Tropical Medicine & Hygiene* **99**:1508-10. Doi: 10.4269/ajtmh.18-0549.

- Esu E, **Tacoli C**, Gai P, Berens-Riha N, Pritsch M, Loescher T & Meremikwu M (2018) Prevalence of the *pfdhfr* and *pfdhps* mutations among asymptomatic pregnant women in Southeast Nigeria. *Parasitology Research* **117**: 801-7. Doi: 10.1007/s00436-018-5754-5.

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- Bélard S, Brand J, Schulze-Sturm U, Janda A; von Both U, **Tacoli C**, Alberer M, Kempf C, Stegemann M, Krüger R, Varnholt V, Blohm M, Reiter K, Zoller T, Suttorp N, Mall M, von Bernuth H, Gratopp A, Hübner J, Hufnagel M, Kobbe R & Kurth F (2019) Intravenous Artesunate for Imported Severe Malaria in Children Treated in Four Tertiary Care Centers in Germany: a Retrospective Study. *Pediatric Infectious Disease Journal* **11**: e295-e300. Doi: 10.1097/INF.00000000000024.

Abbreviations

ACT	artemisinin-based combination therapy
ACD	acid citrate dextrose
AL	arthemeter-lumefantrine
Ala or A	alanine
ANC	antenatal care
aOR	adjusted odds ratio
APC	antigen-presenting cell
AQ	amodiaquine
Arg or R	arginine
ART	artemisinin derivatives
AS	artesunate
AS+SP	artesunate *plus* sulphadoxine-pyrimethamine
AS+AQ	artesunate *plus* amodiaquine
Asn or N	asparagine
Asp or D	aspartic acid
Arps10	apicoplast ribosomal protein S10
BMI	body mass index
CDC	Centers for Disease Control & Prevention
CI	confidence interval
CMC	complete medium for culturing
Corp.	Corporation
CO_2	carbon dioxide
CQ	chloroquine
Cys or C	cysteine
DE	Germany
DHA	dihydro-artemisinin
DHA+PPQ	dihydro-artemisinin *plus* piperaquine
DMSO	dimethyil sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dd	double-distilled
e.g.	*exempli gratia*, for example
ER	endoplasmatic reticulum
et al.	*et alia*, and others
EtBr	ethidium bromide
Fc	final concentration

g	gram or G-force
Gln or Q	glutamine
Gly or G	glycine
GmbH	Gesellschaft mit beschränkter Haftung
GMS	Greater Mekong sub-region
G6PD	glucose-6-phosphate dehydrogenase
h	hour
H_2O	water
Hb	haemoglobin
His or H	histidine
IC_{50}	half maximal inhibitory concentration
ICAM-1	intercellular adhesion molecule-1
i.e.	*id est*, that is
Ile or I	isoleucine
Inc.	Incorporated
IPTp	intermittent preventive treatment in pregnancy
iRBC	infected red blood cell
ITM	Institute of Tropical Medicine
ITN	insecticide-treated net
Kelch13 or K13	Kelch 13 gene
K13	Kelch 13 protein
KDH	Kabutare District Hospital
KEAP1	Kelch-like ECH-associated protein 1
L	liter
Leu or L	leucine
Lys or K	lysine
LLIN	long-lasting insecticide net
LLC.	Limited Liability Company
Ltd.	Limited
m	milli or meter
M	molar or methionine
max.	maximum
Met or M	methionine
$MgCl_2$	magnesium chloride
min.	minute/s or minimum
MMV	Medicines for Malaria Venture
MoH	Ministry of Health
MQ	mefloquine

mut	mutation
n	nano
N_2	nitrogen
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NAI	naturally acquired immunity
NaOH	sodium hydroxide
NEB	New England BioLabs
NFD	N86-184F-D1246 (*pfmdr1* haplotype)
NK	natural killer
nm	nanometer
nM	nanomolar
No. or no.	number
Nrf2	nuclear factor erythroid 2-related factor 2
ns	non-synonimous
O_2	oxigen
OPD	out-patient department
OR	odds ratio
p	p-value
PABA	para-aminobenzoic acid
PCHL	parasite clearance half-life
PCR	polymerase chain reaction
Pf or *pf*	*Plasmodium falciparum*
pfcrt	*P. falciparum* chloroquine resistance transporter gene
PfCRT	*P. falciparum* chloroquine resistance transporter
pfdhfr	*P. falciparum* dihydrofolate reductase gene
PfDHFR	*P. falciparum* dihydrofolate reductase
pfdhps	*P. falciparum* dihydropteroate synthetase gene
PfDHPS	*P. falciparum* dihydropteroate synthetase
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
pfmdr1	*P. falciparum* multidrug resistance 1 gene
PfMDR1	*P. falciparum* multidrug resistance 1
PfMDR2	*P. falciparum* multidrug resistance 2
PfPI3K	*P. falciparum* phosphatidylinositol 3-kinase
Pgh	p-glycoprotein pump
pH	*potential hydrogenii*
Phe or F	phenilalanine
Pibp	phosphoinositide-binding protein

pmol	picomol
Pph	protein phosphatase
PPQ	piperaquine
Pro or P	proline
PRR	pattern-recognition receptor
PtdIns3P	phosphatidylinositol-3-phosphate
Pv or *pv*	*Plasmodium vivax*
pvcrt-o	*P. vivax* chloroquine resistance transporter-orthologous gene
pvmdr1	*P. vivax* multidrug resistance 1 gene
PvMDR1	*P. vivax* multidrug resistance 1
rpm	revolutions *per* minute
RBC	red blood cell
RDT	rapid diagnostic test
RFLP	restriction fragment length polymorphism
RNEC	Rwandan National Ethics Committee
ROSC	reactive oxidative stress complex
RSA	ring-stage survival assay
SEA	Southeast Asia
SEM	standard error of measurement / scanning electon microscopy
Ser or S	serine
SOP	standard operating procedure
SNP	single nucleotide polymorphism
SP	sulfadoxine-pyrimethamine
spp.	species
SSA	sub-Saharan Africa
T_h1	T helper 1
T_h2	T helper 2
Thr or T	threonine
TLR	Toll-like receptor
Tyr or Y	tyrosine
U	unit
UPR	unfolded protein response
uRBC	uninfected red blood cell
US	United States
UTI	urinary tract infection
UV	ultraviolet

V	volt
VCAM-1	vascular cell adhesion molecule-1
Val or V	valine
w/	with
w/o	without
WHO	World Health Organization
x	times
µ	micro
µM	micromolar
°C	degree Celsius
&	and

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Abstract

Malaria is a highly relevant and frequent infectious disease in tropical countries caused by intracellular *Plasmodium* parasites. The worldwide emergence of resistance of *Plasmodium spp.* against available antimalarial drugs such as chloroquine (CQ) and artemisinin derivatives (ART) is a serious threat to malaria control and elimination. Preserving the efficacy of artemisinin-based combination therapies (ACTs) requires a concerted effort at a global level.
In our work, we investigated the geographic extent and prevalence of *P. falciparum* and *P. vivax* drug resistance in Nigeria, Rwanda and south-western India. We assessed the prevalence of polymorphisms in genes involved in drug resistance, namely *P. falciparum K13* (ART)*, pfdhps & pfdhfr* (sulfadoxine-pyrimethamine, SP) *and pfmdr1* (lumefantrine, amodiaquine) as well as *P. vivax* gene *pvmdr1* (CQ) in five field studies conducted between 2010 and 2018 and partially correlated these molecular markers of drug resistance to patients' clinical outcome. Field isolates from Rwanda, taken in 2018, were additionally investigated for their *ex vivo* survival rates upon exposure to ART.
We tracked ART resistance in Rwanda by genotyping the molecular marker *K13* in 360 *P. falciparum* isolates collected in 2010-2018. We showed for the first time that *K13* mutations associated with ART resistance are present there, and thus in Africa, at a low frequency. However, mutations occurred in patients who recovered successfully and/or had *ex vivo* survival rates below the threshold of resistance, thus, their role in ART resistance in the area remains uncertain. Importantly, one patient with high parasite *ex vivo* survival rates but no *K13* mutation did not clear parasitemia after ART treatment. Furthermore, we assessed mutations in *K13, pfdhps*, *pfdhfr* as well as in *pvmdr1* in each ca. 100 *P. falciparum* and *P. vivax* isolates from south-western India. Also, we determined the distribution of *pfmdr1* alleles to appraise the potential of lumefantrine as ACT partner drug. Most of the Indian *P. falciparum* isolates carried mutations conferring mild to severe SP resistance, indicating that the therapeutic lifetime of the leading first-line antimalarial combination SP-artesunate is limited. Due to the predominance of the *pfmdr1* haplotype NFD the use of lumefantrine as alternative to SP is not advised. The virtual absence of the *P. vivax pvmdr1* mutation Y976F and the successful parasite clearance after treatment, indicated that CQ remains effective in vivax malaria in southwestern India. Finally, a high prevalence of *pfdhfr/pfdhps* quadruple and quintuple mutant was observed in Nigerian pregnant women with asymptomatic *P. falciparum* infection, suggesting that its efficacy in preventive intermittent SP treatment in pregnancy might be severely threatened in the country.
These data reflect the abundance of antimalarial drug resistance in different malaria-endemic regions, however, with pronounced differences between regions and drugs. Molecular surveillance of antimalarial drug resistance is a useful approach for detection and potential drug policy changes.

Zusammenfassung

Die Malaria ist weiterhin eine bedeutende Infektionskrankheit in tropischen Ländern. Das weltweite Aufkommen von Resistenzen der Erreger der Gattung *Plasmodium* gegenüber verfügbare Malariamedikamente wie Chloroquin (CQ) und Artemisininderivate (ART) stellen eine ernsthafte Bedrohung für die Malariakontrolle und -bekämpfung dar. Um die Wirksamkeit von Artemisinin-basierten Kombinationstherapien (ACTs) zu erhalten, bedarf es gemeinsamer Anstrengungen auf globaler Ebene.

In unserer Arbeit haben wir die geografische Ausdehnung und Prävalenz von Arzneimittelresistenzen von *P. falciparum* und *P. vivax* in Nigeria, Ruanda und Südwestindien untersucht. Dazu assoziert sind wurden die Prävalenzen von Polymorphismen der Parasiten die mit Arzneimittelresistenz wie *P. falciparum K13*, *dhps*, *dhfr*, *mdr1* und *P. vivax mdr1* in fünf Feldstudien zwischen 2010 und 2018 untersucht und teilweise mit dem klinischen Ergebnis der Patienten korreliert. Bei Feldisolaten aus Ruanda, wurden zusätzlich auf die *ex vivo* Überlebensraten bei Exposition gegenüber ART untersucht.

In Rwanda wurde zwischen 2010 und 2018 der molekularen Marker *K13* bei 360 *P. falciparum* Isolaten genotypisiert, um eine Aussage über die Entwicklung der ART-Resistenz zu treffen. Wir konnten zum ersten Mal zeigen, dass assozierte mit der ART-Resistenz *K13*-Mutationen dort in niedriger Frequenz vorhanden sind. Allerdings traten Mutationen bei Patienten auf, welche sich unter ART-Therapie erfolgreich erholten und/oder bei Isolaten, deren *ex vivo* Überlebensraten auch unterhalb der Resistenzschwelle bleiben, so dass die Rolle der Polymorphismen bei der ART-Resistenz in Rwanda ungeklärt bleibt. Hervorzuheben ist, dass ein Patient mit hohen parasitären *ex vivo* Überlebensraten aber ohne *P. falciparum K13*-Mutation, die Infektion unter Therapie nicht eliminieren konnte. Darüber hinaus untersuchten wir bei ca. 100 *P. falciparum* bzw. *P. vivax* Feldisolaten von 2015 aus Südwest-Indien Mutationen in *P. falciparum* Markern für die Resistenz gegen Sulfadoxin-Pyrimethamin (SP) (d.h. *pfdhps* und *pfdhfr*) und gegen Artesunat (AS) (d.h. *K13*) sowie *P. vivax* Marker für CQ-Resistenz (*pvmdr1*). Zudem haben wir die Allelverteilung von *pfmdr1* bestimmt, um das Potenzial von Lumefantrin als alternatives ACT-Partnermedikament zu bewerten. Die meisten der indischen *P. falciparum* Isolate trugen Mutationen, welche eine leichte bis schwere SP-Resistenz hervorrufen, was darauf hindeutet, dass die therapeutische Lebensdauer der führenden Antimalaria-Kombination AS+SP begrenzt ist. Aufgrund der Dominanz des *pfmdr1*-Haplotyps NFD ist die Verwendung von Lumefantrin als Alternative zu SP nicht zu empfehlen. Die faktische Abwesenheit der *pvmdr1*-Mutation Y976F und die erfolgreiche Parasitenbeseitigung nach der Behandlung zeigten, dass CQ der Therapie der Malaria tertiana im Studiengebiet weiterhin wirksam ist. Bei nigerianischen Schwangeren mit asymptomatischer *P. falciparum* Infektion wurde eine hohe Prävalenz von *pfdhfr/pfdhps* Vier-

und Fünffachmutanten beobachtet. Dies weist darauf hin, dass Wirksamkeit der intermittierender, preventiver Therapie Schwangerer mit SP in Nigeria ernsthaft gefährdet ist. Diese Daten spiegeln die Häufigkeit der Resistenzen gegen Malaria-Medikamente in verschiedenen endemischen Regionen wider, jedoch mit ausgeprägten Unterschieden zwischen Regionen und Medikamenten. Die molekulare Überwachung der Resistenz gegenüber Malariamedikamenten ist ein nützlicher Ansatz zur frühzeitiger Erkennung, die ihrerseits die Bedeutung für die potentielle Änderung der Behandlungsrichtlinien.

1. Introduction

1.1. Malaria

Malaria remains the most dangerous vector-borne infectious disease in tropical and sub-tropical countries, causing 219 million clinical episodes and 437,000 deaths only in 2017 (WHO, 2018).

The impressive global decrease of malaria incidence observed since 2010 has been considered one of the greatest achievements of the public health sector of the past decade. Nevertheless, data from the period 2015-2017 highlight a troubling shift in the disease trend. Progress appeared to have stalled and, in a few countries and regions, a reversal in the gains achieved has been reported (WHO, 2018).

In 2016, an estimated 216 million cases of malaria occurred worldwide, an increase of 5 million cases over the previous year. Even though since 2000 the improvements in prevention and control measures have led to a global reduction in malaria mortality, nowadays still more than 3 billion people – almost half of world's population – are at risk of contracting malaria (Figure 1.1). The most affected regions remain in sub-Saharan Africa and in India, which bear over 80% of the world malaria burden (WHO, 2018). Of note, in 2017 five countries accounted for nearly half of global malaria cases: Nigeria (25%), Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%).

The prevalence of malaria in endemic regions depends on both envonmental and socio-economic factors including the distribution of asymptomatic carrier reservoirs, hygiene conditions, measures for control and prevention, access to healthcare, rainfall intensity and treatment availability, among others.

Children under five years of age show increased vulnerability to the disease, due to their immature immune system. In 2017, children younger than five years of age accounted for 61% of global malaria deaths. Another subgroup at high risk is represented by pregnant women (especially primigravidae) due to their insufficient degree of immunity against placenta-specific parasite strains. Over 32 million pregnant women are estimated to be at risk of *P. falciparum* malaria in sub-Saharan Africa annually (Dellicour *et al.*, 2010). Malaria adversely affects the outcome of pregnancy. The fetus is at increased risk of premature delivery and stillbirth, intrauterine growth retardation, low birth weight, anaemia and congenital malaria (Brabin, 1991, Desai *et al.*, 2007, McGready *et al.*, 2012).

Infections in adults from highly endemic areas are often mild or asymptomatic and consequently fail to be treated. This reservoir of untreated individuals promotes the seasonal propagation of *Plasmodium* infections especially in Africa (Chen *et al.*, 2016).

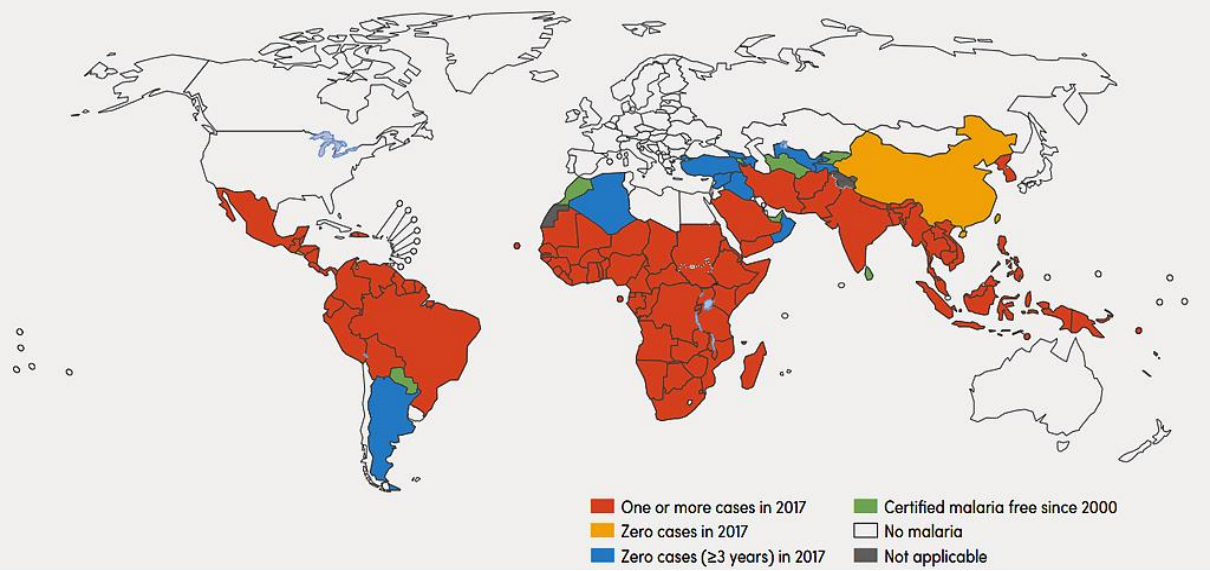

Figure 1.1: Prevalence and distribution of malaria cases in 2017. Close to half of the population in the world was at risk of malaria in 2017. Countries with indigenous malaria cases are colored in dark red. Countries with zero indigenous cases over the past 3 consecutive years (light blue) are today considered malaria-free. Figure adapted from WHO malaria report 2018.

1.2. *Plasmodium* parasite

Malaria is caused by an obligate intracellular protozoan parasite of the genus *Plasmodium* that belongs to the Apicomplexa phylum. It comprises over 120 species, of which only five cause the disease in humans, *i.e.*, *Plasmodium malariae*, *Plasmodium ovale, Plasmodium knowlesi*, *Plasmodium vivax,* and *Plasmodium falciparum* (Garnham, 1966, Ahmed & Cox-Singh, 2015).

The timing that incurs between two peaks of fever is among the distinguishing feature between *Plasmodium* species (Greenwood *et al.*, 2005).

Among these species, *Plasmodium falciparum* and *Plasmodium vivax* account for more than 95% of all human malaria infections and thus are a major public health concern (Larson, 2019). *P. falciparum* represents the most virulent species, and is the most prevalent malaria parasite on the African continent. It is responsible for most malaria-related deaths globally (Bhatt *et al.*, 2015), while *P. vivax* is the most widespread human malaria parasite outside of the African continent, putting 2.5 billion people at risk of infection and causing 25-40% of global malaria cases every year (Gething *et al.*, 2012, Howes *et al.*, 2016).

Morevover, *P. vivax* and *P. ovale* may give rise to new waves of disease due to the awakening of parasites in a dormant state called hypnozoites that are able to survive for long periods of time in the liver of infected individuals. *P. malariae* does not form hypnozoites but it is able to survive for long periods in an asymptomatic blood stage (Greenwood *et al.*, 2008).

1.2.1. *Plasmodium* life cycle

Malaria is transmitted by *Anopheles* mosquitos, which are endemic in 95 countries. Human infection takes place during the blood meal of the female mosquito when hundreds of uninucleate sporozoites are ejected from the mosquito's salivary glands into the human bloodstream (Figure 1.2). Once in the host, sporozoites rapidly infect liver cells where they undergo nuclear division and mature into liver-stage schizonts. Schizonts lyse, releasing 2,000 to 40,000 merozoites into the bloodstream where they quickly invade erythrocytes starting the so-called erythrocytic cycle (Dvorak *et al.*, 1975). The *Plasmodium* hepatocytic (exoerythrocytic) cycle is asymptomatic since only a small number of liver cells is infected by the parasites. During this hepatocytic phase, *P. vivax* and *P. ovale* sporozoites can transform in hypnozoites, the reactivation of which gives rise to malaria relapses after months (MMV, 2014).

The merozoites, which have invaded the red blood cells (RBCs) develop into trophozoites, a metabolically active stage. The asexual multiplication of *Plasmodium* within the infected RBCs (iRBCs) leads to the formation of erythrocytic schizonts that eventually cause the lysis of the infected erythrocyte and the further release of 16-32 merozoites into the bloodstream to infect other RBCs (Cowman & Crabb, 2006). The erythrocytic cycle takes 24 hours in case of *P. knowlesi* infections, 48 hours in *P. falciparum, P. vivax* and *P. ovale* infections and 72 hours in case of *P. malariae*. The rupture of the infected erythrocytes triggers the release of pyrogens causing the typical periodic fever of malarial infections.

In this phase, *Plasmodium spp.* evade the human immune system mainly by avoiding exposure, *i.e.*, replicating within the iRBC into a parasitophorous vacuole, and by infecting only erythrocytes that (unlike other cells) lack the MHC class I presentation system and therefore cannot be targeted by conventional T cells.

The high virulence of *P. falciparum* has been attributed to its capability of invading all RBC age classes achieving high parasite densities, while the other human *Plasmodium* species, which are more discerning in the RBCs they invade, are relatively more benign disease agents (Kerlin & Gatton, 2013). Furthermore, *P. falciparum* mediates iRBCs cytoadhesion and sequestration in the vascular system. Intraerythrocytic parasites remodel the host cell surface, exporting several proteins to take up nutrients and to dispose waste for their maturation. Since modified iRBCs are recognized and cleared by the spleen, *P. falciparum* late developmental stages (trophozoites and schizonts) express a protein called PfEMP1 that mediate iRBCs adhesion to the vascular endothelium (cytoadherence). Such attachment favours binding with and accumulation of other iRBCs (sequestration). Sequestration of iRBCs in the blood vessels is a major contributor to mortality and morbidity in falciparum malaria. For instance, parasite sequestration in the microcapillaries of the brain causes vascular occlusion leading to local hypoxia and inflammation typical of cerebral

malaria (Cunnington *et al.*, 2013). Also, *P. falciparum* parasite harbours a family of 60 *var* genes encoding a wide range of PfEMP1 surface proteins thus slowing down the potential acquisition of immunity. During the erythrocytic phase, few parasites develop into mature sexual stages, termed female and male gametocytes that will be taken up with the ingested blood during a next mosquito blood meal.

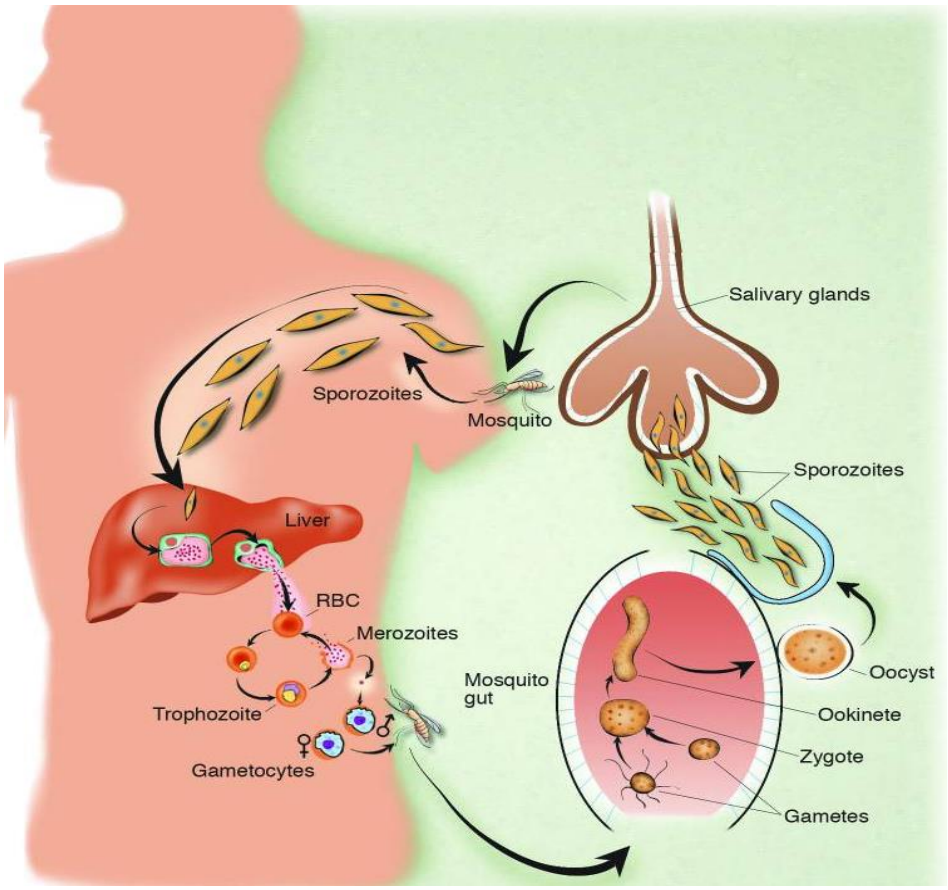

Figure 1.2: The life cycle of malaria parasite. It includes 3 cycles: the sporogonic cycle in the mosquito vector, the hepatic and erythrocytic cycles in the human host (White, 2004).

Once in the mosquito gut, male and female gametocytes differentiate becoming fully functional gametes. Once mature, gametes fuse forming a diploid zygote. The zygote elongates and becomes able to move (ookinete). It travels across the midgut wall and turns into a spherical oocyst. Inside the oocyst, the nucleus divides repeatedly and gives rise to numerous sporozoites that develop until oocyst rupture (8-16 days according to *Plasmodium* species). Sporozoites migrate from the mosquito gut to the salivary glands ready for a new infection (Guttery *et al.,* 2015, Sinden, 2015).

Among the 400 species of *Anopheles* identified throughout the world, only 60 are potential vectors for the parasite (Tuteja, 2007). In Africa, the main anopheline vectors are represented by *Anopheles gambiae* and *Anopheles funestus*. In India, six taxa are major malaria vectors with regional distribution, including *Anopheles stephensi*, *a* vector species of urban malaria (Dev & Sharma, 2013).

1.3. Human host

To date, the complex interplay of parasite components with the host immune system is an object of in-depth study, especially for the development of an effective vaccine against the disease.

Host immunity is an important determinant of antimalarial drug efficacy, influencing the outcome of prevention measures and treatment. Immunity against malaria can be classified into innate immunity and naturally acquired immunity. The innate antimalarial immunity is intrisically present in the host and is not dependent on any previous infection. It is an inherent genetic refractoriness towards *Plasmodium* infection or towards the development of severe symptoms that include alterations in the structure of host haemoglobin or in certain enzymes as described in the next section. Natually acquired immunity (NAI) is developed by people living in highly endemic areas with stable malaria transmission (Perlmann & Troye-Blomberg, 2002, Kumar *et al.*, 2007). These individuals acquire the capacity to overcome the negative effects caused by the pathogen by blocking the progression of the disease and its clinical manifestation (Doolan *et al*., 2009). This type of partial immunity will be further discussed in section 1.3.2.

1.3.1. Host genetics

Plasmodia have played a key role during the evolution of the genus Homo, progressively influencing its development. Human populations affected by malaria since ancient time have biologically adapted by developing specific genetic disorders. For instance, it has been proven that haemoglobinopathies characterized by the presence of haemoglobin C or S affect *Plasmodium* ability to develop within the iRBCs during the erythocytic cycle, protecting the human host from severe malaria (Allison, 1954, Chakravorty & Williams, 2015, Lee & Hufford, 1990). In the same way, there is strong evidence that glucose-6-phosphate dehydrogenase (G6PD) deficiency or α-thalassemia confer protection against malaria (Flint *et al.*, 1986, Luzzatto & Bienzle, 1979, Tishkoff *et al.*, 2001).

The high prevalence of certain genetic conditions in malaria endemic regions underlines how these variations have prevailed as a consequence of natural selection due to malaria over time. This is particularly true in the African continent, where the strong selective pressure caused by the predominant *P. falciparum* parasite has led to a spread of heterozygous carriers of sickle cell-trait (Allison, 1954b, Piel *et al.*, 2010).

Furthermore, African populations lack the Duffy antigen required for the invasion of RBCs by *P. vivax* and are therefore considered resistant to vivax malaria. In contrast, Duffy positive phenotypes are common in several regions of Asia and Latin America (Miller *et al.*, 1976).

1.3.2. Naturally acquired immunity

Continued exposure to malaria parasites induces the development of partially protective immunity in adults. These semi-immune individuals can suppress clinical symptoms also in the presence of otherwise considerable parasitaemia (a phenomenon called anti-disease immunity) and are able to activate a prompt, non-specific immune response against acute malarial infections that prevent the development of severe disease (Carter & Mendis, 2002, Doolan *et al.*, 2009). This type of naturally acquired immunity (NAI) or semi-immunity typically develops in areas of high *P. falciparum* transmission, and it remains to date poorly understood (CDC, 2018).

The adaptive immunity activated by blood-stage malaria parasites relies on humoral and cell-mediated responses (Langhorne, 1989, Playfair *et al.*,1985, Dick *et al.*, 1996). In response to parasite antigene recognition by pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and CD36, dendritic cells mature and migrate to the spleen, the primary site of immune response to the intraeythrocitic parasite. Maturation of dendritic cells is associated with the upregulated expression of MHC class II molecules and the production of cytokines which activate natural killer (NK) cells. NK cells produce interferon-γ (IFN-γ), inducing T helper 1 (T_h1) $CD4^+$ cells differentiation. Additional signals provided by co-stimulators expressed on antigen-presenting cells (APCs) such as dendritic cells promote T cell activation (Figure 1.3). The best-defined costimulatory pathways are the B7:CD28 pathway and the CD40:CD40 ligand (CD40L) pathway (Lenschow *et al.*, 1996, Grewal *et al.*, 1998). In addition, the NKT cell subset has been shown to promote *P. falciparum*-infected erythrocyte lysis and to be a potent inhibitor of liver-stage parasite replication in mouse models (Manoor *et al.*, 2002). T_h1 cells activate macrophage iRBC phagocytosis, and the consequent production of cytokines and inflammatory molecules such as nitric oxide and oxygen radicals.

The contemporary activation of T helper 2 (T_h2) $CD4^+$cells leads to the onset of humoral immune responses. Malaria gives rise to strongly elevated blood concentrations of non-malaria-specific immunoglobulins, but the importance of the related polyclonal B cell activation in NAI remains unclear. In the early 60s, a study reported that γ-globulins from adults in highly malaria endemic areas were able to protect children against malaria (Cohen *et al.*, 1961). Later on, the PfEMP1 protein was identified as a major target for protective antibodies in individuals from high transmission areas (Bull *et al.*, 1998).

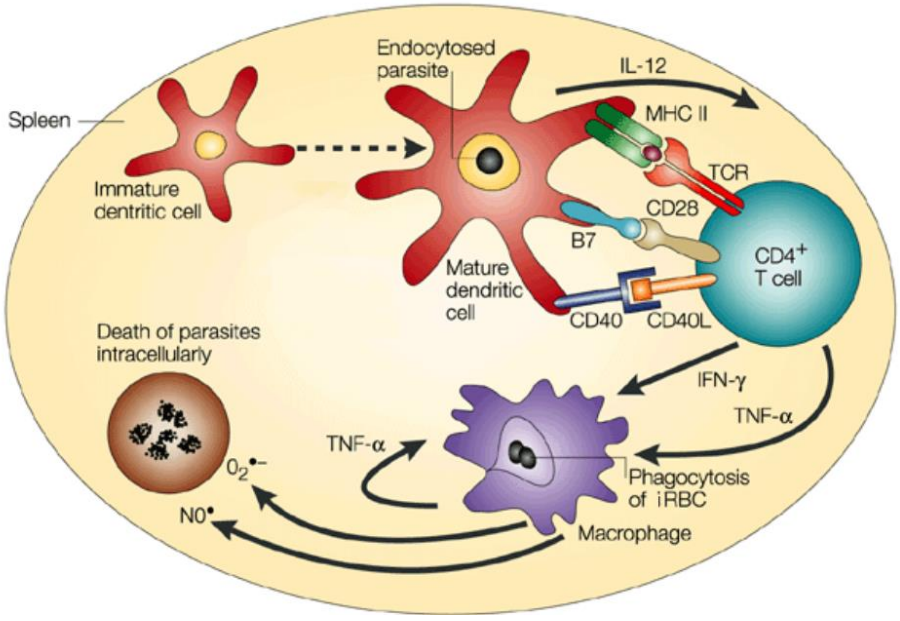

Figure 1.3: T cell immunity against malaria: Activation of $CD4^+$ T cells by mature dendritic cells leading to macrophage activation, phagocytosis of parasitized red blood cells and elaboration of cytokines and small inflammatory molecules (such as nitric oxide and oxygen radicals). Figure adapted from Good, 2001.

The immune response triggered by *Plasmodium* blood stage infection is a double-edged sword for the host since the over-production of IFN-γ and of the tumor nectosis factor-α (TNF-α) upregulates the expression of major PfEMP1 protein binding receptors such as ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) enhancing iRBC sequestration in the blood vessels (Berendt *et al.*, 1989, Lennartz *et al.*, 2017, Mahamar *et al.*, 2017). It has been proposed that $CD8^+$ T cells regulate immunosuppression in acute malaria and down-modulate inflammatory responses but as human erythrocytes do not express MHC antigens their role against blood-stage parasites is still uncertain (Miyahira *et al.*, 1998, Perlmann & Troye-Blomberg, 2002).

A better understanding of NAI mechanisms would shed a light on why factors such as age, host genetics, pregnancy, HIV co-infection, intensity and seasonality of malaria transmission, can positively or negatively affect cure rates following treatment (Rogerson *et al.*, 2010). In 2002, Mannoor *et al.* firstly hypothesized that primordial, extrathymic T cells as well as autoantibody-producing B-1 cells could have been the prime movers of NAI response. Researchers showed that infected mice encountered thymic atrophy during the infection with consequent arrest of thymic T cells differentiation and high activation of extrathymic T cells in the spleen and liver. Furthermore, athymic mice were able to survive malaria when infected with a reduced number of parasitized RBC thanks to a NKT cells subset of non-thymic origin (Mannoor *et al.*, 2002).

A different form of acquired immunity towards *Plasmodium* infections called premunition can develop in response to repeated infections. Premunition or concomitant

immunity is a state where the host is protected from further infection with a given species by ongoing persistent infection with the same organism. This infection-immunity protects against hyperparasitaemia without eliminating the infection. It is acquired relatively rapid, but it is lost just as quickly when the individual is no longer over-exposed (Ryan *et al.*, 2010, Mouchet *et al.*, 2008).

1.3.3. Clinical malaria

The presence of several parasite strains at a certain geographical location and the wide spectrum of antigenic variants occurring during infection in areas with stable and intense *falciparum* malaria transmission promote the early acquisition of an immune response in the host (section 1.3.2.). However, naturally acquired immunity is slowly developed and remains species-specific and strain-specific. Newborns from semi-immune mothers are protected during the first few months of life by maternal antibodies transferred through the placenta. As antibodies decrease with time, this passive immunity is followed by 1-2 years of increased susceptibility before the acquisition of effective immunity. The risk of contracting clinical disease is already high at around 3 to 4 months of age (depending on transmission rate) when infants become susceptible to severe disease and death (CDC, 2018).

Uncomplicated malaria symptoms appear flu-like: chills, fever, general indisposition, abdominal pain, sweat, headache, nausea, vomiting, and mild anaemia. In young children, malaria may also present with lethargy, loss of appetite and cough. When the disease is cured at this early stage with appropriate antimalarial drugs, death occurs in less than 0.1% of cases. If not treated or treated with ineffective and poor-quality medicines, the uncontrolled *P. falciparum* parasite multiplication leads to a severe form of the disease characterized by vital-organ dysfunction, impairment of consciousness, metabolic acidosis, problems in breathing, severe anaemia and at worst cerebral malaria, coma and death. The risk of cerebral malaria is particularly high in children of 2-4 years of age. In endemic areas where each individual is exposed to repeated infections, children reach a protective semi-immune status between 5-15 years of age. Over this period, the frequency of clinical disease begins to diminish, and the risk of mortality strongly decreases. In these areas, most inhabitants generally possess NAI by adulthood (CDC, 2018).

Fatality associated with untreated severe *P. falciparum* malaria can reach 50% and can occur within the first 24 hours from presentation (White, 2004, Zaloumis *et al.*, 2014). As expected, uncomplicated (and often asymptomatic) malaria seems to affect mostly adults from endemic areas probably as a result of their state of NAI/premunition while severe malaria is mostly reported in children, in pregnant women, in immune-compromised individuals and in non-immune adults (WHO, 2014).

1.4. Fight against malaria: chemotherapy & chemoprophylaxis

In recent years, the magnitude of the economic and public health burden that malaria imposes on endemic countries as well as the failure of global eradication campaigns have strongly renewed the interest in malaria control and elimination strategies.

Malaria control measures traditionally rely on case management and on mosquito vector control achieved by removal of breeding sites and by the use of insecticides and of insecticide-treated bed nets (ITNs). Since an effective and universally available licensed malaria vaccine is still missing, in spite of the strong efforts of the past six decades in this direction, chemotherapy and chemoprophylaxis remain of vital importance in the fight against malaria.

The antimalarial drugs administered to cure and prevent *Plasmodium spp.* infections are classified based on the parasite life cycle stage they affect (Figure 1.4). The class of chemotherapeutic agents mostly in use act against the asexual erythrocytic stages. These erythrocytic schizonticides usually clear parasitaemia within a few days and include artemisinin derivatives among others (Delves *et al.*, 2012). Hypnozoiticides are prescribed to eliminate *P. vivax* and *P. ovale* hypnozoites from the liver to prevent a relapse of malaria. They are generally administered together with tissue schizonticides which prevent the development of liver-stage parasites (Kumar *et al.*, 2003). Gametocytocidal compounds inhibit the transmission of the parasite to the *Anopheles* mosquitoes (Schlitzer, 2007) while few compounds termed sporontocides are available to inhibit the formation of oocysts and sporozoites in the anopheline vector to prevent the spread of malaria to humans.

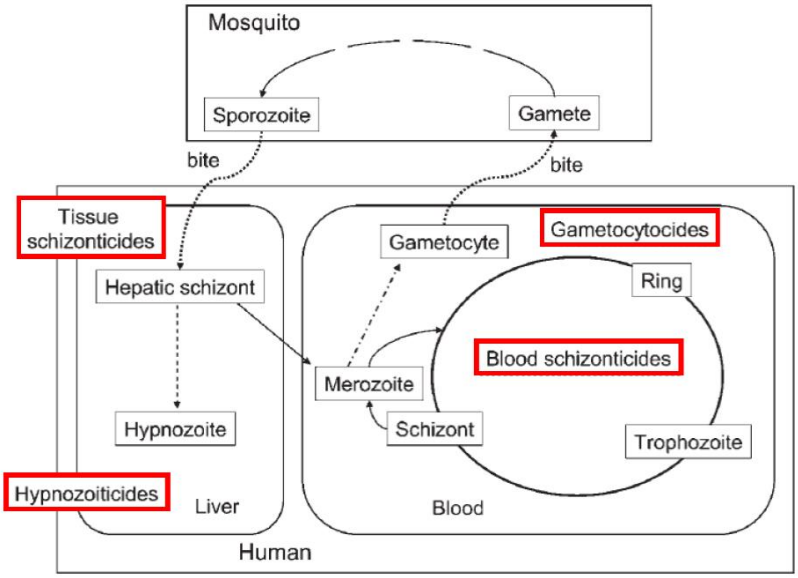

Figure 1.4: Main classes of antimalarial compounds according to their parasite cycle stage of action. Blood schizonticides act on the erythrocytic phases of parasite development and are widely used (Schlitzer, 2008).

In our studies, we aimed to obtain a snapshot of the global extent of antimalarial drug resistance. Therefore, in the next section a brief description of the main classes of antimalarial drugs will be provided, with special emphasis on the ones mainly under focus in this thesis, *i.e.*, chloroquine, sulfadoxine-pyrimethamine and artemisinin derivatives.

1.4.1. Major antimalarial drugs

Chemotherapy mainly focus on *P. falciparum,* the most lethal of the plasmodia.
The main classes of compounds used are:

Aminoquinolines: are 4-aminoquinoline derivatives used against plasmodial erythrocytic stages and include amodiaquine, chloroquine and hydroxychloroquine. Chloroquine for decades has been the drug of choice for the treatment of uncomplicated malaria and for chemoprophylaxis. Nowadays, it is still recommended for first-line treatment of vivax malaria in most endemic countries. Its mode of action will be described in detail in the next section.

Inhibitors of the parasite respiratory chain: such as atovaquone that irreversibly binds to the mitochondrial cytochrome bc1 complex of the parasite, blocking electron transport. Its toxicity appears to be selective for the parasite mitochondria, as the host mitochondrial function is not affected by atovaquone therapy (Korsinczky *et al.*, 2000). Nevertheless, adverse effects such as agranulocytosis and hepatitis have been observed upon use of this drug (Bloland & Williams, 2002). To avoid the onset of resistance, atovaquone is prescribed in combination with proguanil (Srivastava *et al.*, 1999, Vaidya & Mather, 2000).

Antibiotics: act on the protein synthesis machinery of the mitochondrion (or apicoplast) (Vaidya, 2004, Goodman *et al.*, 2007) killing the parasite in the second cycle of erythrocytic replication (Dahl *et al.*, 2006, Ramya *et al.*, 2007). The effect of the antibiotic treatment is slow (Lell & Kremsner, 2002). Therefore, they are prescribed as monotherapy only for prophylaxis and in combination with faster drugs for malaria treatment. Best known antimalarial antibiotics are doxycyclin, clindamycin and azithromycin.

Antifolates: are drugs that target intermediates of folic acid metabolism, a compound required for DNA biosynthesis in the parasite. This class of antimalarials includes a variety of dihydrofolate reductase inhibitors (such as chlorproguanil, proguanil, pyrimethamine, and trimethoprim) and sulfa-drugs (sulfalene, sulfamethoxazole, dapsone and sulfadoxine among others). A typical combination is sulfadoxine-pyrimethamine, that will be further discussed in section 1.4.1.2.

Artemisinin derivatives: are semi-synthetic derivatives that were firstly developed in order to improve the solubility and overcome pharmacokinetic problems specific to artemisinin. They represent the most potent antimalarial drugs available today and are

recommended for first-line treatment of falciparum malaria worldwide. Their molecular structure and mechanism of action will be detailed in section 1.4.1.3.

1.4.1.1. Chloroquine (CQ)

Chloroquine (CQ) is a 4-aminoquinoline derivative of quinine which accumulates in the digestive vacuole of the parasite during its erythrocytic phase interfering with haemoglobin metabolism. The intraerythrocytic parasite uses host haemoglobin (Hb) as its primary food source. About 60% – 70% of host infected erythrocyte's Hb is taken up by the parasite and degraded into the digestive vacuole to give amino acids for parasite's protein synthesis (Figure 1.5) (Foley & Tilley 1997, Egan, 2003).

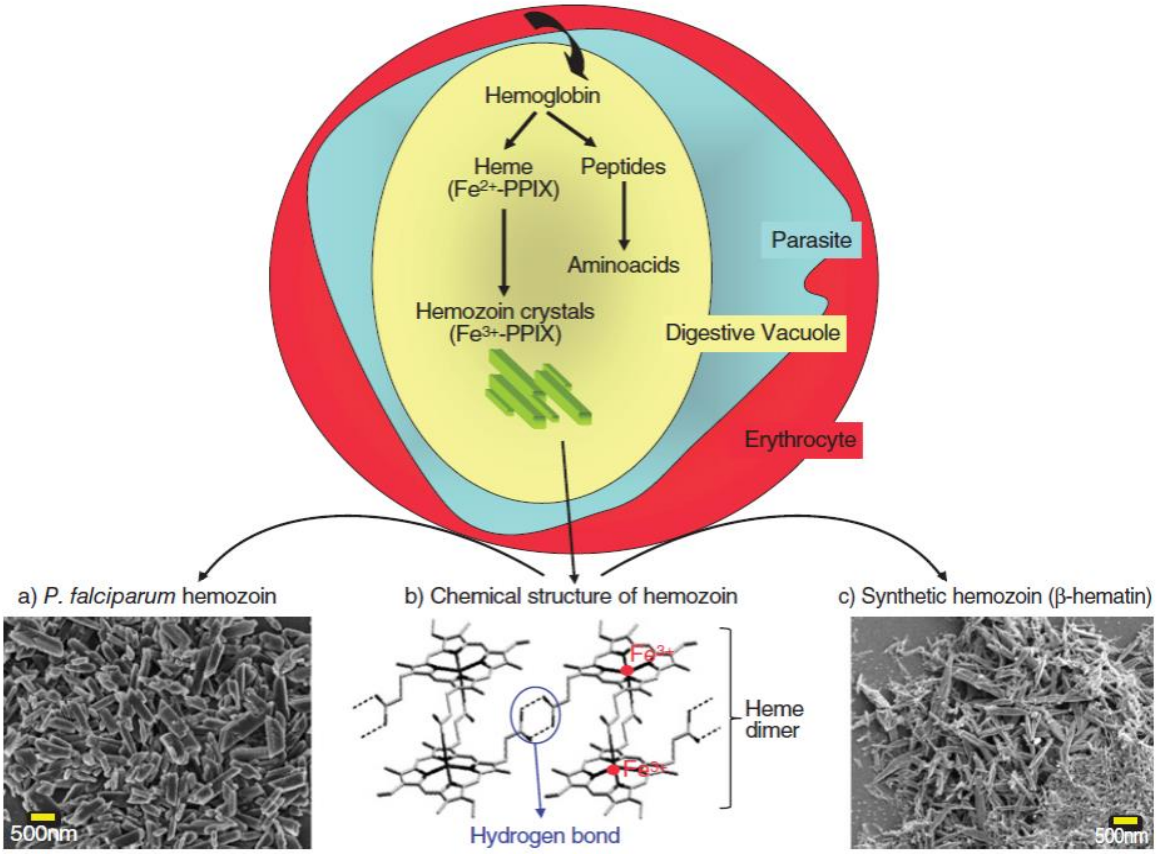

Figure 1.5: Haemoglobin degradation and hemozoin formation in the digestive vacuole of *Plasmodium falciparum*. The free heme released by haemoglobin degradation is detoxified by converting it into insoluble crystals of hemozoin. (**a**) SEM picture of purified natural hemozoin from *P. falciparum*. (**b**) Chemical structure of hemozoin crystals. (**c**) SEM picture of synthetic hemozoin crystals (Coban *et al.*, 2010).

The heme group released during Hb digestion is converted into hematin by reaction with molecular oxygen and with dismutated superoxide ions. Even though hematin is relatively insoluble in aqueous solution, it can diffuse through the lipidic membranes where it undergoes

peroxidation, killing the parasite. To prevent this process, the parasite has developed a detoxification pathway that converts the monomeric hematin into a compact and insoluble crystal called hemozoin (Egan, 2008, Egan, 2008b). CQ accumulates into the parasite's digestive vacuole where it forms π-π bonds with hematin avoiding its bio-crystallization in hemozoin and killing the parasite (Fitch, 2004).

CQ has been the perfect drug for treating acute malaria in endemic settings. It was cheap, universally effective against all plasmodia species, deliverable over a brief period in few doses, and safe even for pregnant women and children and came with few side effects (Cooper & Magwere, 2008). Unfortunately, over the past 60 years the onset and spread of CQ resistance in *P. falciparum* parasites has prevented its use for falciparum malaria treatment, but it remains today the therapy of choice for radical cure of vivax malaria worldwide.

1.4.1.2. Sulfadoxine-pyrimethamine (SP)

Sulfadoxine and pyrimethamine, the constituents of *e.g.*, Fansidar ®, are folic acid antagonists. Folic acid is a vital nutrient for *Plasmodium* cell growth and reproduction since it is required for the synthesis, repair and methylation of DNA. Sulfadoxine (as all sulfa-drugs) is an antimetabolite, *i.e.*, it competes with para-aminobenzoic acid (PABA) for incorporation into folic acid. In particular, sulfadoxine inhibits the parasite enzyme dihydropteroate synthetase (DHPS) which is necessary for the conversion of PABA to folic acid. Pyrimethamine blocks the activity of the enzyme dihydrofolate reductase (DHFR), inhibiting the biosynthesis of purines and pyrimidines. The lack of nitrogenous bases prevents DNA synthesis and cell multiplication in both parasite intra- and exoerythrocytic cycles blocking schizont formation (Law *et al.*, 2014, Wishart *et al.*, 2017).

To date, sulfadoxine-pyrimethamine (SP) is one of most widely used antimalarial combinations in the world and is recommended for preventive treatment of malaria in pregnancy. Intermittent preventive treatment in pregnancy (IPTp) with SP is recommended in all African areas with moderate and high transmission of malaria. Treatment should start as soon as the woman enters the second trimester of pregnancy and should include at least 3 doses of SP (each of 1500 mg sulfadoxine/75 mg pyrimethamine) given at 1 month apart (WHO, 2018c). The SP treatment clears both existing infections that consist of drug-sensitive parasites (treatment effect) and prevents incident infections (prophylactic effect) (ter Kuile *et al.,* 2007). The increase of SP resistance in stable malaria transmission areas has compromised the beneficial effects of IPTp-SP and will be further discussed in section 1.5.1.2.

1.4.1.3. Artemisinin derivatives (ART)

Chemically, artemisinin derivatives (ART) are sesquiterpene lactones with an endoperoxide bridge that is essential for their antimalarial activity (Meshnick, 2002, Schlitzer, 2008). Dihydro-artemisinin (DHA) which is the active metabolite of all ART, has served as a template for the synthesis of a series of semisynthetic analogues such as artemether and artesunate, which are the most effective antimalarial drugs in use to cure uncomplicated and severe falciparum malaria (Bray *et al*., 2005, Liu *et al.*, 2011).

The mechanism of action and the intracellular targets of artemisinin compounds have been for long an important subject of study. Hb digestion plays a crucial role in ART activity providing a likely explanation for the decrease of sensitivity towards these drugs in metabolically inactive early ring-stage parasites (Meshnick *et al.*, 2002, O'Neill *et al.*, 2010).

The bio-activation of the ART endoperoxide bridge is promoted by the ferrous iron mainly produced during hemozoin reduction to heme. According to Vaid *et al.*, the endoperoxide bridge opening generates cytotoxic radical intermediates that react with susceptible targets such as phosphoinositides involved in the regulation of signaling and trafficking events in the parasite (Vaid *et al.*, 2010). In particular, ART are effective inhibitors of *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K), an enzyme involved in the endocytosis of iRBC cytoplasm (hence of host Hb) into the digestive vacuole of the parasite. The inhibition of PfPI3K causes the entrapment of Hb in vesicles within the parasite cytoplasm and therefore arrests parasite replication and growth (Mbengue *et al.*, 2015).

However, proteomic studies reveal that there are probably hundreds of targets, and thus, ART-mediated killing may be due to a more generalized alteration of proteins' structure and functionality (proteopathy) (Takala-Harrison *et al.*, 2015, Tun *et al.*, 2015). In a recent study, an engineered artemisinin-based activity probe was capable of binding a multitude of intracellular targets (mostly enzymes) involved in key metabolic pathways of the parasite including the metabolism of carboxylic acids, cellular biogenic amines and nucleosides and ribonucleoside biosynthesis (Wang *et al.*, 2015) (Figure 1.6).

ART have a great potential as antimalarial drugs. Artesunate is administered intravenously (IV) or intramuscularly (IM) for the treatment of severe malaria caused by *P. falciparum* and is particularly valuable (as other ART) when used in combination with additional antimalarials for oral therapy. Artemisinin-based combination therapy (ACT) is currently recommended as first-line medication for the treatment of uncomplicated falciparum malaria and CQ-resistant *P. vivax* infections and will be further discussed in the next session.

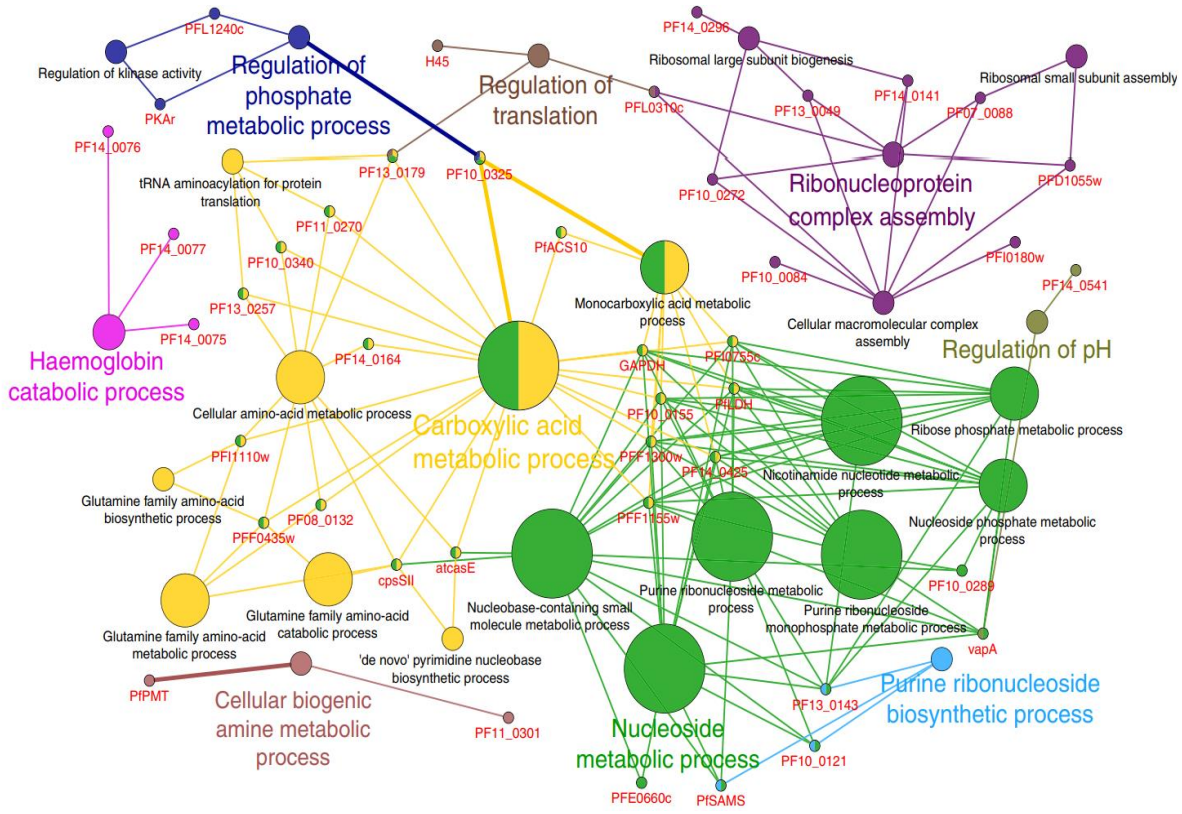

Figure 1.6: ART targets are potentially involved in several essential biological processes of *Plasmodium falciparum*. The biological pathways of the parasite affected by ART-probe are highlighted in different colors (Wang *et al.*, 2015).

1.4.2. Artemisinin-based combination therapy (ACT)

Malaria treatment has changed substantially in the past two decades. Intravenous artesunate has proven to be superior to quinine in treating severe falciparum malaria (Dondorp *et al.*, 2005, Kurth *et al.*, 2015) and regimens known as artemisinin-based combination therapy (ACT) demonstrate greater efficacy in treating uncomplicated *P. falciparum* malaria cases, reducing transmission in endemic areas and lowering the levels of reinfection (Bouchaud *et al.*, 2012, Pousibet-Puerto *et al.*, 2016). ACTs combine a fast-acting ART with a partner drug that has a longer half-life. DHA is the main metabolite of artemisinins and the fastest acting antimalarial available, reducing parasite burden of up to 10,000-fold every 48 h (Tilley *et al.*, 2016). ART inhibit the development of the erythrocytic trophozoites, killing circulating parasites before they get sequestered in the deep microvasculature. Additionally, ART reduce gametocytogenesis preventing malaria transmission and spread (Mehra & Bhasin, 1993).

Since ART have an elimination half-life of only approx. 45 min., they are co-administered with longer half-life partner drugs, such as lumefantrine, amodiaquine (AQ), piperaquine (PPQ), mefloquine (MQ), sulphadoxine-pyrimethamine (SP) or pyronaridine. The

administration of ACT provokes the rapid reduction of parasitaemia during the first three days of treatment (reduction of parasite biomass) followed by clearance of the residual parasites by the partner drug.

1.5. Antimalarial drug resistance

Case management has relied largely on antimalarial medicines thanks to their affordability, availability and effectiveness. Together with antipyretics, antimalarials are the most widely used drugs in the Tropics. Nevertheless, their extensive usage over the past six decades especially as monotherapies has posed an intense selective pressure on malaria parasites leading to the emergence of resistance.

Antimalarial drug resistance, particularly in *Plasmodium falciparum,* has been a major contributor to the resurgence of malaria worldwide (Marsh, 1998). Resistance has most likely provoked the strong increase in malaria-related child mortality in Africa observed at the beginning of the century (Korenromp *et al.*, 2003) and could be responsible for the new trend towards increase in malaria cases observed in recent years (WHO, 2018).

Introduced in 1946, CQ has been the first safe, effective and economic antimalarial compound, and it is nowadays still recommended as first-line treatment for *P. vivax*, *P. ovale* and *P. malariae* infections (WHO, 2001). *P. falciparum* CQ-resistant variants were firstly observed at the Thai-Cambodian border in late 1950s (Harinasuta *et al.*, 1965) and later reported in South America (Payne, 1987). In the 1970s, CQ resistance had spread worldwide (with large differences in its degree) and the drug combination SP began to be administrated for the treatment of falciparum malaria in Southeast Asia, South America and parts of Africa (Harinasuta *et al.,* 1967, Laing, 1968, Black *et al.*, 1981, Nurse, 1981).

In the 1990s, as a response to increasing levels of *P. falciparum* antimalarial resistance, the WHO recommended that all countries experiencing resistance to conventional monotherapies, such as CQ and SP, would start using combination therapies containing ART such as artesunate and arthemeter for falciparum malaria.

Nevertheless, in 2008-2009, the promising role played by ACTs had been compromised by the onset of ART resistance in Southeast Asia and today five countries in the Greater Mekong sub-region are confirmed to have a stable ART-resistant parasite population, *i.e.*, Cambodia, Myanmar, Vietnam, Thailand and Lao People's Democratic Republic (WHO, 2018b) (Figure 1.7). In these countries, there are five ACTs which are currently approved and recommended by WHO, and a sixth one (artesunate-pyronaridine) is currently under evaluation (WHO, 2018b).

For what concerns *P. vivax* infections, the first case of CQ resistance has been observed in 1989 in Papua New Guinea (Rieckmann *et al.,* 1989). Sporadic cases of CQ

treatment failure in vivax malaria have later been reported in Indonesia and in the Philippines (Baird *et al.*, 1991, Murphy *et al.*, 1993, Barnadas *et al.*, 2008) as well as in a few countries of southeast Asia such as India, Myanmar and Vietnam (Dua *et al.*, 1996, Guthmann *et al.*, 2008, Phan *et al.*, 2002) and in South America (Phillips *et al.*, 1996).

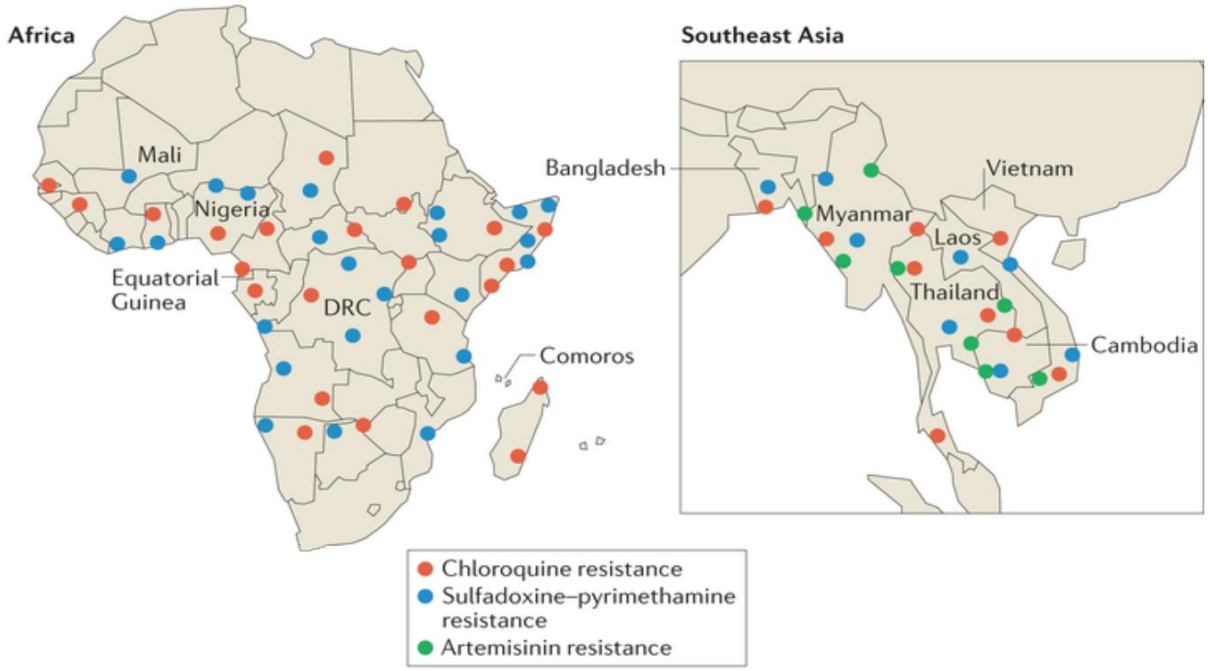

Figure 1.7: Spread of antimalarial drug resistance. Detailed maps showing the distribution of *P. falciparum* resistance to chloroquine, sulfadoxine-pyrimethamine and artemisinin in Africa and Southeast Asia. Figure adapted from Haldar *et al*., 2018.

1.5.1. Genetic determinants of resistance

Point mutations and/or changes in the copy number of genes encoding for drug targets occur spontaneously, affecting parasite susceptibility towards antimalarials. Antimalarial targets are mostly enzymes involved in essential metabolic pathways or influx/efflux pumps that affect intra-parasitic concentrations of the drug. Increasing levels of resistance can develop as consequence of a single genetic event or it can be multigenic (White, 2004).

1.5.1.1. CQ resistance associated genes

CQ resistance in *P. falciparum* results from multiple genetic events. Initial point mutations in the gene encoding for the transporter PfCRT (*Plasmodium falciparum* chloroquine resistance transporter) at position 76 (Lys→Thr) has been observed in virtually all resistant field isolates

(with one reported exception of a 76A variant by Chaijaroenkul *et al.* in 2011). To date, no less than 30 substitutions have been identified, making PfCRT an incredibly polymorphic protein (Ecker *et al.*, 2012).

PfCRT variants prepare the ground for mutations in a second transporter called PfMDR1 (*Plasmodium falciparum* multidrug resistance 1), which encodes an energy-demanding p-glycoprotein pump (Pgh). PfMDR1 has been shown to modulate parasite susceptibility *in vitro*, but its role in CQ treatment failure remains uncertain (Djimdé *et al.*, 2001, Plowe, 2003). Of note, non-synonymous single nucleotide polymorphisms (nsSNPs) in PfMDR1 especially at codon 86, 184 and 1246 have been associated with the development of resistance in few partner drugs used in currently recommended ACTs such as amodiaquine (AQ) and lumefantrine (Table 1.1).

Increased risk of lumefantrine treatment failure *in vivo* (Venkatesan *et al.,* 2014) and reduced parasite susceptibility *in vitro* are associated with specific alleles of *pfmdr1* such as the haplotype N86-184F-D1246 (Mwai *et al.*, 2009, Nsobya *et al.*, 2010). Resistance to mefloquine has been associated with increased *pfmdr1* gene copy number (duplication, not mutation) (Price *et al.*, 2004).

The cause of CQ resistance in *Plasmodium vivax* has not been elucidated yet. Despite its association to substantial morbidity (Genton *et al.*, 2008, Tijitra *et al.*, 2008), *P. vivax* infections have received little attention and limited funds for research and control, since they usually produce less severe symptoms than falciparum malaria (Mendis *et al.*, 2001, Anstey *et al.*, 2009, Mueller *et al.*, 2009).

	Lumefantrine	Amodiaquine	Chloroquine	Mefloquine
pfmdr1 N86Y	N86	86Y	86Y	
pfmdr1 Y184F	184F	Y184		
pfmdr1 D1246Y	D1246	1246Y		
pfmdr1 copy no.				↑ Increased
pfcrt K76T	K76	76T	76T	

Table 1.1: nsSNPs and copy number variations associated with resistance to ACT partner drugs. Modified from Otienoburu *et al.*, 2019.

Furthermore, the exclusive parasite tropism for reticulocytes (erythrocyte precursors) rather than mature RBCs impedes the development of an *in vitro* culture of *P. vivax* (Bermùdez *et al*., 2018) and hence, the clear identification of molecular markers of resistance. In addition, the *in vivo* relapses of hypnozoite stages and recrudescences complicate the estimation of

the clinical efficacy of CQ. Today, the most reliable proof of CQ resistance is given when circulating parasites are detected in the peripheral blood in the presence of therapeutic chloroquine concentrations (*i.e.*, >100 ng/mL) (Phyo & Nosten, 2018).

However, two genes orthologous to *pfmdr1* and *pfcrt*, which encode the putative transporters *pvmdr1* and *pvcrt-o,* respectively, have been identified as potential genetic markers of CQ resistance in *P. vivax.* In Southeast Asia (SEA), mutant alleles of both genes have been associated with CQ resistance *in vivo* and *in vitro* (Brega *et al.*, 2005, Lu *et al.*, 2011). Particularly, a substitution at codon 976 (Tyr→Phe) of the *pvmdr1* gene has been associated with reduced CQ sensitivity in a few studies in SEA in particular in Thailand, Myanmar and Indonesia (Imwong *et al.*, 2003, Suwanarusk *et al.*, 2008, Nyunt *et al.*, 2017). Moreover, in an *in vitro* susceptibility assay performed by Suwanarusk and co-workers, *P.vivax* isolates carrying mutation Y976F had significantly higher 50% inhibitory concentration (IC_{50} values) upon exposure to CQ as compared to wildtypes (Suwanarusk *et al.*, 2007). At last, sextuple and septuple *pvmdr1* mutants carrying both Y976F and a substitution at position 513 (Ser→Arg) were identified in Madagascar during a study on CQ therapeutic efficacy that resulted in 5% treatment failure (Barnadas *et al.*, 2008). However, current knowledge on the distribution of these mutations and of the respective haplotypes remains scarce, especially in India that alone accounts for almost half of global vivax malaria cases (WHO, 2018).

1.5.1.2. Molecular markers of SP resistance: *pfdhfr* & *pfdhps*

Sulfadoxine-pyrimethamine (SP) was introduced as first-line treatment of uncomplicated falciparum malaria in 1973 in Palin, at the Thai-Cambodian border, due to the emergence of CQ resistance. The development of SP resistance at this same location occurred around 1980 as consequence of several factors including the wide availability and unrestricted usage of the drug combination, the poor compliance (especially among migrants) and the distribution of pyrimethamine as medicated salt, as a general fever remedy and for chemoprophylaxis (Verdrager, 1986).

P. falciparum SP resistance is associated with the acquisition of polymorphisms in the parasite genes *pfdhps and pfdhfr*, encoding for the enzymes dihydropteroate synthase and dihydrofolate reductase of parasite folic acid synthesis pathway, respectively (section 1.4.1.2.). The nsSNPs observed at these genetic loci have been proven to alter the affinity of the proteins they encode towards the drug but not the enzymes functionality (Yaro, 2009). In particular, a substitution at codon 108 in PfDHFR is known to cause the aminoacid substitution Ser→Asn conferring a certain degree of pyrimethamine resistance (Foote *et al.*, 1990, Basco *et al.*, 1996, Reeder *et al.*, 1996). Moreover, reduced susceptibility *in vitro* to the drug has been reported as a consequence of additional point mutations at residue 51, causing Asn→Ile,

59 (Cys→Arg), and/or 164 (Ile→Leu) (Sirawaraporn *et al.*, 1997, Sirawaraporn *et al.*, 1997b). Today, 67% of parasites sampled in Thailand, Cambodia, and Myanmar carry the I164L mutation in *pfdhfr* (Nair *et al.*, 2003). Further mutations associated with pyrimethamine resistance are A16V, C50R, S108N/T and V140L (Peterson *et al*., 1990, Plowe *et al.*, 1997). Analysis of microsatellite markers has shown that single (S108N) and double (S108N *plus* N51I or S108N *plus* C59R) PfDHFR mutants have developed rapidly in Asia after SP introduction and later spread to Africa (Roper *et al.*, 2004).

For what concerns the development of resistance against sulfadoxine, several nsSNPs in the coding region of *pfdhps* reduce enzyme affinity towards sulfa-drugs. Particularly, mutations at codon 436 (Ser→Phe), 437 (Ala→Gly), 540 (Lys→Glu), 581 (Ala→Gly) and 613 (Ala→Ser or Thr) are associated with *in vitro* sulfadoxine resistance of *P. falciparum* (Wang *et al.*, 1995, Basco & Ringwald, 1998). Transfectional studies (Triglia *et al.,* 1998, Kublin *et al*., 2002) have demonstrated that point mutations in PfDHPS confer resistance to the whole class of sulfa-drugs. Other *pfdhps* nsSNPs affecting *P. falciparum* susceptibility to this class of drugs are I431V, S436A/F, and K540E. The mutation at codon I431V is novel and its association to antifolate resistance is still poorly understood. However, it has been detected in concurrence with mutation A437G, A581G and A613S in Nigerian isolates and in Cameroon were *pfdhfr/pfdhps* alleles formed the octuple mutant N51I-C59R-S108N/I431V-S436A-A437G-A581G-A613S (Triglia & Cowman 1994, Sutherland *et al.*, 2009, Chauvin *et al.*, 2015).

1.5.1.3. *Kelch13*: a molecular marker of ART resistance

In 2014, a gene located on the chromosome 13 called *Kelch13* or more simply *K13,* was identified as a useful molecular marker for tracking the emergence and spread of ART-resistant *P. falciparum* parasites (Ariey *et al.,* 2014).

K13 encodes for a 726 amino acid protein which contains a BTB/POZ domain and a C-terminal Kelch motif made of 6 blade-shaped β-sheets (also called Kelch domains) that form the protein β-propeller domain. Mutations in the C-terminal β-propeller have been associated with delayed parasite clearance following ART treatment in SEA (Ariey *et al*., 2014, Ashley *et al.*, 2014). K13 is predicted to be a regulator of protein quality control. The Kelch motif shares some (25–30%) sequence identity with a sub-class of human Kelch-like proteins called KLHL, which are widely distributed throughout eukaryotes (Dhanoa *et al.*, 2013). KLHL proteins mediate protein degradation by interacting with a Cullin-E3 ligase and with the specific substrate to polyubiquitinate. Cullin-E3 ligase coordinates an E2 ubiquitin-conjugating enzyme which promotes substrate degradation by the ubiquitin-proteasome system. In the same way, it has been hypothesized that the BTB/POZ domain of K13 binds to a Cullin-E3 ligase, and the C-terminal propeller domain act as substrate adaptor, binding to specific target

proteins to accelerate their ubiquitylation and proteasomal degradation (Figure 1.8a) (Ding *et al.*, 2008). K13 ubiquitylation activity is further supported by evidences that selective inhibitors of the plasmodial proteasome and generic proteasomal inhibitors work synergistically with ART and other antimalarial compounds enhancing parasite killing rates (Prasad *et al.*, 2013, Dogovski *et al.*, 2015, Li *et al.*, 2016).

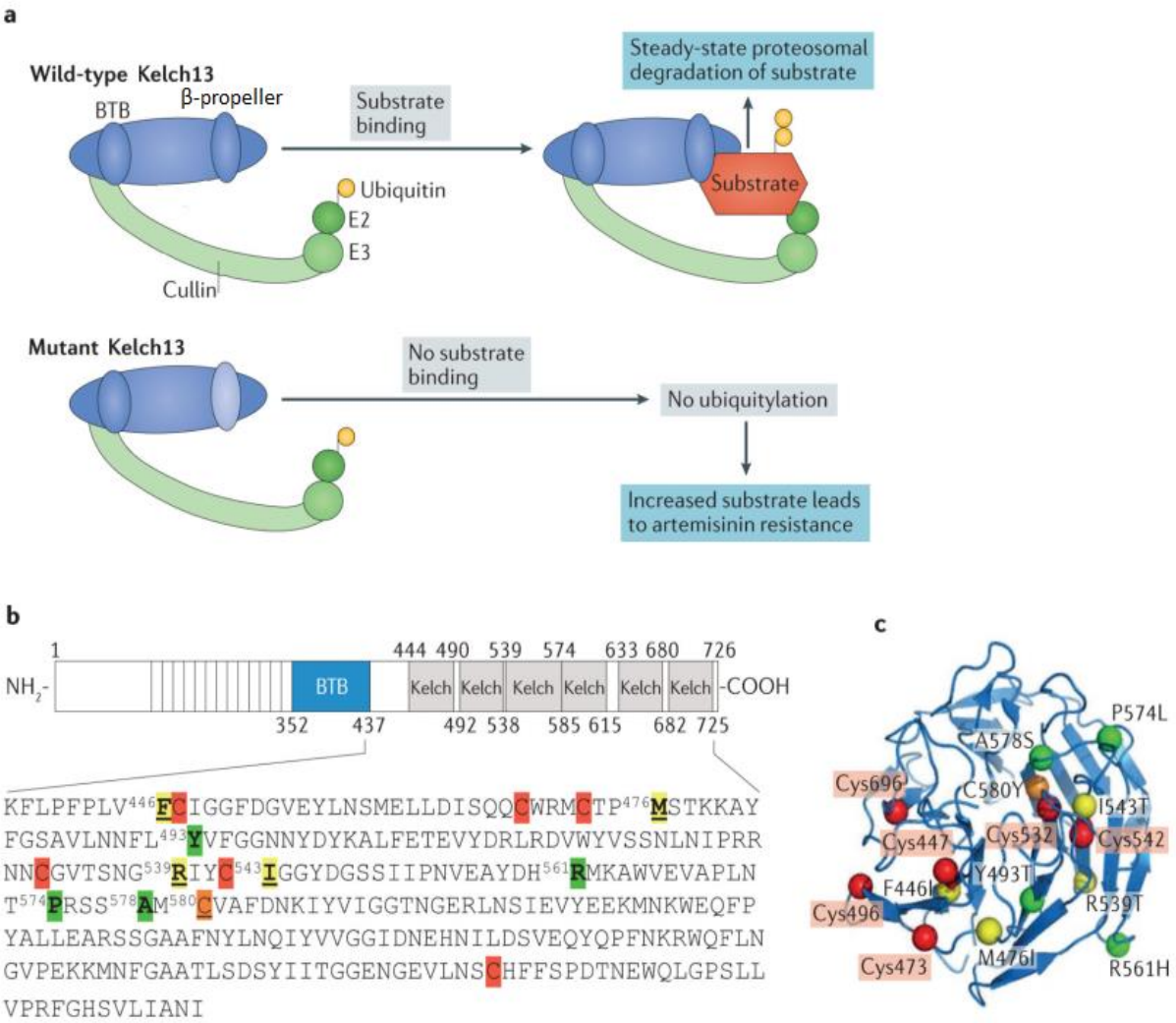

Figure 1.8: Kelch13 (K13) structure and putative function.(**a**) K13 is thought to be a substrate adapter for the E3-E2 ubiquitination system. The BTB/POZ domain of K13 binds to a Cullin-E3 ligase and the C-terminal β-propeller domain coordinates the specific substrate to ubiquitylate. Mutations in the β-propeller's Kelch domains reduce substrate binding activity. (**b**) Amino acid sequences of K13 β-propeller domain. The BTB domain and the six Kelch domains of the C-terminal are highlighted in blue and grey, respectively. Cysteine residues are indicated in red (Cys447, Cys469, Cys473, Cys532, Cys542 and Cys696). Mutation C580Y (shown in orange) is the major mutation associated with ART resistance in Cambodia. Mutations known to confer ART resistance such as R539T, I543T, F446I and M476I are indicated in yellow. Mutations that are not associated with ART resistance (*i.e.*, A578S) or that still need to be validated, *e.g.,* R561H, Y493T and P574L are indicated in green. (**c**) A 3D model of K13 amino acid residues 338–726 showing the position of validated and candidate mutations associated with ART resistance. Of note, validated mutations occurred in proximity to cysteine residues and primarily in β strands while candidate mutations are present on the loops. Figure adapted from Haldar *et al.*, 2018.

Nevertheless, the actual substrate (or substrates) of K13 is still unknown and could be involved in one or more pathways of resistance.

The process of recovery from ART-induced proteopathy involves multiple cellular functions including the removal of misfolded, altered proteins and toxic protein aggregates and the production of a new protein milieu. K13 may play a role in restoring such complex system of protein functions (so-called proteostasis) in the parasite by taking part in the activation and stimulation of the unfolded protein response (UPR) involved in the cellular cytoprotective mechanism (Mok *et al.*, 2015). The function of the UPR is to re-establish endoplasmatic reticulum (ER) homeostasis by overcoming imbalanced protein-folding capacity mainly through the proteosomal activity of the reactive oxidative stress complex (ROSC) and to initiate a transcriptional response aimed at stimulating gene expression and restore redox conditions. In eukaryotic species, the UPR activates the nuclear factor erythroid 2-related factor 2 (Nrf2), a central transcriptional factor for cytoprotection against stress. Nrf2 activates nearly 600 cytoprotective target genes and it is negatively regulated by the Kelch-like ECH-associated protein 1 (KEAP1) (Cullinan *et al.*, 2003).

Due to its structural analogy with KEAP1, K13 is thought to be involved in UPR's transcriptional repression. Under unstressed conditions, Nrf2 is constantly ubiquitinated and degraded in proteasomes. Exposure to oxidative stress promotes the modification of cysteine residues, or of residues close to them, in the KEAP1 primary structure which in turn reduces the Nrf2 presentation rate for ubiquitination. The over-produced Nrf2 relocates in the nucleus where it activates the transcription of the genes involved in the cellular anti-oxidant response. In this same way, K13 may impair its interaction with a transcription regulator involved in the *P. falciparum* cytoprotective response (Paloque *et al.*, 2016). The putative role of K13 as a redox sensor is supported by the presence of six cysteine residues in the β-propeller domain as observed in KEAP1 (which harbours seven). Mutations in the β-propeller are predicted to induce conformational changes that decrease the affinity of K13 for its target substrate, potentially altering both the levels and location of the target protein or target proteins (Haldar *et al.*, 2018) (Figure 1.8b and 1.8c).

Another potential mechanism of ART resistance in *P. falciparum* parasites is the proteostatic dysregulation of *P. falciparum* phosphatidylinositol 3-kinase (PfPI3K) which stimulates the production of the lipid phosphatidylinositol-3-phosphate (PtdIns3P) (Mbengue *et al.*, 2015). K13 could promote poly-ubiquitination (and thus degradation) of PfPI3K. Immunoprecipitation assays have proven that wildtype K13 binds PfPI3K but its affinity to this substrate strongly reduces when harboring mutation C580Y, which is the major mutation responsible for clinical ART resistance in SEA. The overproduction of PfPI3K and consequent increase in the levels of the lipid product PtdIns3P in parasites carrying K13 variants could enable ring-stage parasites to survive ART-mediated inhibition of PfPI3K. Increased production of PtdIns3P has

been observed also in isolates harbouring the Cambodian mutation associated to ART resistance R539T (Mbengue *et al.*, 2015).

Although the effects of most K13 mutations on PfPI3K binding remain unclear, the prevalent Cambodian mutations associated with ART resistance, *i.e.*, C580Y, R539T and I543T as well the major Myanmar mutation F446I and the laboratory-generated substitution M476I are located within 1–3 amino acids of a cysteine residue, whereas candidate mutations whose correlation to ART resistance still has to be proven such as Y493T, R561H and P574L or polymorphisms not associated with resistance (A578S) are located in distal loop regions (Figure 1.8b and 1.8c, Haldar *et al.*, 2018). Nevertheless, this mechanism of action of K13 still awaits confirmation since it is unlikely that the modest (2 to 4-fold) increase in PfPI3K levels observed in K13 mutant parasites could lead alone to the high resistance observed in ring-stage parasites with mutant K13 *in vivo* upon exposure to ART (>70–fold) (Dogovski *et al.*, 2015).

To date, 13 non-synonymous *K13* polymorphisms (*i.e.*, P441L, F446I, G449A, N458Y, Y493H, R539T, I543T, P553L, R561H, V568G, P574L, C580Y and A675V) have been reported to be associated with clinical ART resistance in SEA (Ménard *et al.,* 2016). According to population studies, *K13* variant C580Y is predominant in Cambodia, Myanmar and eastern and western Thailand, accounting for 80% of resistant cases (Ariey *et al.*, 2014, Ashley *et al.*, 2014, Talundzic *et al.*, 2015, Wang *et al.*, 2015b). In Vietnam, mutations C580Y, P574L, V568G, P553L, I543T and Y493H have been recorded from the southern part of the country, on the border with Cambodia (Takala-Harrison *et al.*, 2015). In Africa, *K13* polymorphisms are also observed (Taylor *et al.,* 2015, Conrad *et al.*, 2014), but their role in ART treatment failure is still under evaluation (Figure 1.9).

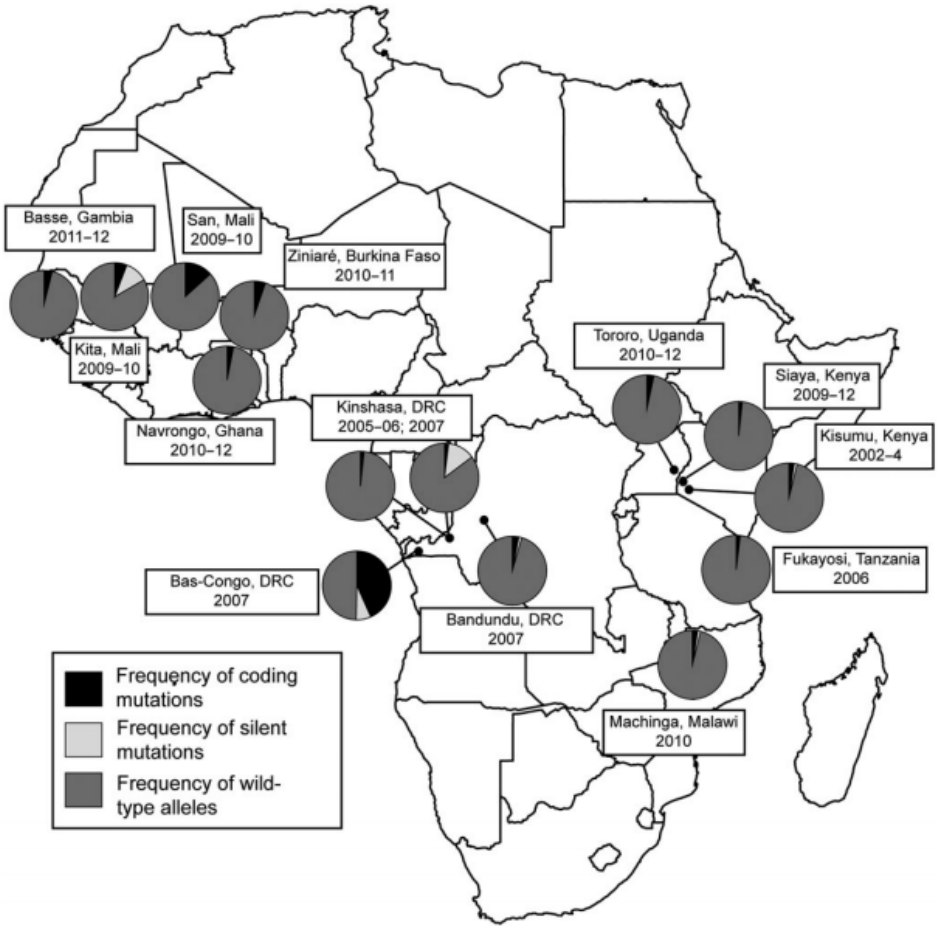

Figure 1.9: Distribution of *P. falciparum K13*-propeller mutations in 14 sub-Saharan African sites. The proportions of wildtype alleles, coding mutations, and silent mutations within each geographic site are highlighted in dark grey, light grey and black, respectively. Abbreviation: DRC, Democratic Republic of the Congo (Taylor *et al.,* 2015).

1.5.2. ART resistance *in vivo* and *in vitro*

The first case of reduced susceptibility to ART *in vivo* was reported in 2007 in Pailin, western Cambodia (Dondorp *et al.*, 2009). Few years later, parasites with prolonged clearance time were further reported at other Cambodian locations as well as in Thailand and in Vietnam (Ariey *et al.*, 2014, Winzeler & Manary 2014). In the following decade there has been a continued increase in reported cases and range of infections with apparently ART-resistant parasites (Noedl *et al.*, 2008, WHO, 2013) and today ART resistance extends across the Greater Mekong subregion (GMS) in SEA from the eastern coast of Vietnam to the Indian sub-continent (Tun *et al.*, 2015, Grist *et al.*, 2016).

ART resistance in *P. falciparum* lengthens the parasite clearance half-life (PCHL) during therapy. For this reason, the accurate measurement of PCHL *in vivo* is recommended to assess therapeutic responses to ART in falciparum malaria. The standard method used to

analyze parasite clearance curves relies on the calculation of PCHL as a plot of frequent parasite density counts in malaria patients with initial parasitaemia of 10,000 parasites/μL of blood or greater against the time after starting treatment (Flegg *et al*., 2011) (Figure 1.10).

This measure is particularly useful in assessing ART-containing regimens, as these drugs have a much greater effect on circulating ring-stage parasites than do other antimalarials in current use (White, 2017). ART resistance manifests as loss of ring stage susceptibility and is indicated by a PCHL value above 5 hours. Importantly, the delayed clearance of the parasite upon exposure to ART does not necessary lead to treatment failure and is therefore considered a partial resistance. Treatment failures reported in the GMS have occurred exclusively when partial ART resistance was in combination with resistance to the ACT's slow-acting partner drug (WHO, 2018b).

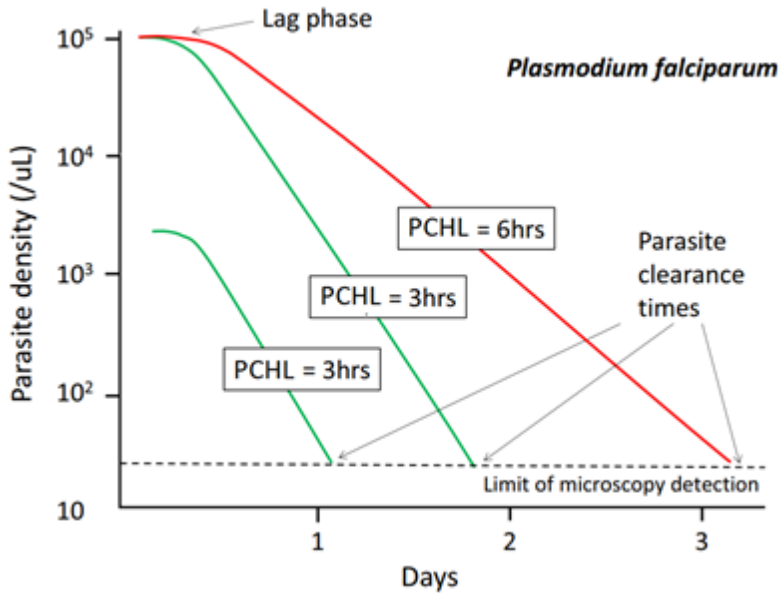

Figure 1.10: Parasite clearance during the 3 days ACT regimen in falciparum malaria. After an initial and variable lag phase, which depends on the stage of parasite development, the decline in parasitaemia is generally log linear. Two infections with ART-sensitive *P. falciparum* parasites (showed in green) had a 50-fold difference in initial parasitaemia which resulted in an 18 h difference in parasite clearance time but presented the same rate of parasitemia decline, *i.e.*, the same clearance half-lives (3 h). The ART-resistant infection (in red) showed a PCHL value of 6 h. Figure adapted from White, 2017.

PCHL assessment has been used widely as an outcome measure in clinical trials and for epidemiological assessments of ART efficacy (Dondorp *et al.*, 2009). Nevertheless, these studies are logistically and financially challenging since they require the screening of a great number of febrile individuals over entire high-transmission seasons to enrol the few patients (in low transmission areas <5% over thousands of enrolled patients) who meet inclusion criteria and agree to several days of hospitalisation.

Since the first report of ART clinical treatment failure, several attempts to develop an *in vitro* assay to assess ART resistance have been done. However, most studies aimed at testing *in vitro* susceptibility by continuous exposure of *P. falciparum* isolates to low levels of dihydro-artemisinin (DHA) during the parasite entire erythrocytic cycle and never consistently correlate with *in vivo* clinical efficacy (Noedl *et al*., 2008, Dondorp *et al.*, 2009, Amaratunga *et al.*, 2012, Teuscher *et al.*, 2012, Klonis *et al*., 2013). More in detail, the reason why the IC_{50} value observed in culture (*i.e.*, the drug concentration required to inhibit parasite growth by 50%) did not correlate to high PCHL observed *in vivo,* was that the parasites were exposed to very low concentrations of DHA for 48–72 h, whereas parasites *in vivo* were exposed to much higher concentrations of DHA for only 1–2 h. On this assumption, Witkowski and co-workers developed in 2013 the first assay designed to reliably correlate *in vitro P. falciparum* susceptibility with *in vivo* parasite clinical phenotype *i.e.,* parasite clearance rates during ACT (Witkowski *et al.*, 2013). The reliability of this assay, called ring-stage survival assay (RSA), was proven in a study on 31 Cambodian patients where all isolates classified as ART-resistant by *in vivo* parasite clearance assessment (*i.e.*, PCHL >5 h) also had e*x vivo* survival rates greater than 10% by RSA (Witkowski *et al.,* 2013). The RSA principle and procedure will be described in details in section 2.7.5.6.

A PCHL cut-off of 5 hours is currently being used to define the ART resistance phenotype in SEA *in vivo* while the RSA is an optimal reference assay to define this same phenotype *in vitro*. Nevertheless, PCHL and RSA are best used to confirm putative resistance and for research, while determination of residual parasitaemia on Day-3 of ACT regimens is a more suitable tool for surveillance and monitoring of resistance onset. According to WHO guidelines, routine monitoring of therapeutic drug efficacy should include the determination of Day-3 parasite density and the assessment of treatment failure rates in clinical trials with follow-up of 28 to 42 days (depending on the ACT in use). A proportion of treatment failure over 10% will determine the change of national antimalarial treatment policy (Tilley *et al.*, 2016).

Suspected partial ART resistance occurs when at least one of the following conditions applies: i) ≥5% of patients carry candidate *K13* mutations associated with ART resistance, ii) ≥10% of patients show persistent parasitaemia on Day- 3 or iii) ≥10% of patients show a clear PCHL >5 h post treatment with an ACT or ART monotherapy. Additionally, ≥10% prevalence of any *K13* mutations could indicate the emergence of a clonal ART-resistant parasite population and should be kept under surveillance (WHO. 2018b). When resistance is suspected by the observation of condition ii) and iii), *K13* marker analysis should be performed urgently, *e.g.*, on parasite DNA extracted from filter paper blood spots. New *K13* variants should be validated *in vitro* by RSA or transfection assay.

Confirmed partial ART resistance is defined by a concurrence of the above-mentioned conditions, *i.e.*, ≥5% of patients carrying clinically or genetically validated *K13* mutations, with

these patients being parasitemic on Day-3 of ART treatment or having a prolonged clearance half-life over 5 hours.

K13 mutations associated with ART resistance can be candidate or validated mutations according to the folling criteria: i) a statistically significant association ($p < 0.05$) on a sample of at least 20 clinical malaria cases between the *K13* variant in analysis and PCHL over 5 h or Day-3 parasitaemia, or ii) >10% RSA survival rates in at least 5 individual isolates with a given *K13* mutation or a statistically significant difference ($p < 0.05$) in RSA survival rates between culture-adapted recombinant parasite lines transfected to express *K13* variant alleles as compared to wildtype. When both conditions i) and ii) are met, the *K13* variant is a validated mutation associated with ART resistance while if only one condition is satisfied (i or ii) mutations are considered candidate (WHO. 2018b).

1.6. Malaria: state of art in endemic areas

Today malaria remains a major health issue in tropical and subtropical countries. In the 50s, the disease was already eradicated in the United States and in more than two dozen countries in Europe, the Americas and Asia as part of the Global Malaria Eradication Program mainly by spraying homes with organochlorine insecticides such as DDT, by draining mosquito breeding sites and by widespread usage of the cheap and effective drug chloroquine. Most of Africa was excluded from the program due to logistical difficulties (WHO, 2016b, Snow *et al.*, 2017).

Malaria incidence strongly depends on the environmental suitability for *Anopheles* vectors (*i.e.*, altitude, vegetation, temperature, humidity, precipitation) and on the implementation of control measures, it is therefore linked to both climate change and poverty. Vertical transmission (from mother to child), or via blood transfusion, are less common but still pose a significant risk in low-income settings (Abdullah & Karunamoorthi, 2016).

Although insecticide-treated bed nets (ITNs) ownership has steadily increased since 2010 in sub-Saharan Africa, where ITNs are the main method of vector control, malaria prevention and control efforts are threatened by the widespread resistance of malaria vectors to the pyrethroids used in ITNs and to multiple classes of insecticides including DDT. Furthermore, in sub-Saharan Africa, intermittent preventive treatment of malaria in pregnancy (IPTp) with SP is recommended to all pregnant women entering the second trimester. In 2018, IPTp-SP coverage was variable among the 39 African countries that have adopted this policy, but only in Zambia half of the pregnant women attending antenatal care visit were receiving at least 3 of the four doses of SP recommended (WHO, 2018).

P. falciparum accounts for the almost totality of estimated malaria cases in the African continent that alone bears more than 90% of global malaria morbidity (200 million malaria

cases reported in Africa only in 2017) (WHO, 2018). *P. faciparum* is further responsible for the majority of malaria cases reported in the WHO regions of Southeast Asia (62.8%), in the eastern Mediterranean regions (69%) and among the western Pacific area (71.9%) (WHO, 2019). Although ACTs in Africa remain highly effective (98-99.5%), arthemeter-lumefantrine (AL) treatment failure rates greater than 10% were reported in Malawi and in Gambia in 2010 and in Angola in 2013 and 2015, jeopardising the future use of this ACT in these regions.

Furthermore, artesunate *plus* sulphadoxine-pyrimethamine (AS+SP) treatment failures has been reported in Somalia and Sudan between 2011 and 2015, causing the change of treatment policies to AL and dihydro-artemisinin-piperaquine (DHA-PPQ). Studies on AS+SP efficacy in India in 2012 have led to a change in drug treatment policy to AL in the north-eastern part of the country that will be further discussed in section 1.6.2.

Globally, in 2017 the 3.4% of estimated malaria cases were caused by *P. vivax*, with 56% of these cases being in the WHO Southeast Asia Region. Treatment of *P. vivax* infections with chloroquine (CQ) followed by primaquine remains the most used therapy wordwide although high CQ treatment failure rates have been reported in a few countries including Myanmar. Of note, no resistance towards ART has been reported in *P. vivax* so far. While *P. vivax* and *P. falciparum* have similar rates of frequency and distribution in Asia and Oceania, in Central and South America the number of vivax malaria cases doubles the number of *P. falciparum* infections (WHO, 2018). *P. vivax* malaria is less common in Africa thanks to the prevailing Duffy antigen negativity in the population (section 1.3.1.). *P. malariae* and *P. ovale* have a wide distribution but low incidence, with *P. ovale* mainly being found in Africa and Southeast Asia. *P. knowlesi*, known to infect long-tailed and pig-tailed macaques, has been the causative agent of several human deaths in SEA, especially in Malaysia (Ahmed & Cox-Singh, 2015).

In the following sections, a more detailed description of the past and current challenges in malaria control, prevention and therapy faced by the Indian population and the African populations of Nigeria and Rwanda will be provided for a better understanding of the aim of the present work.

1.6.1. Nigeria

The Democratic Republic of the Congo and Nigeria together account for more than 35% of global falciparum malaria deaths (WHO, 2018). In Nigeria, malaria incidence is estimated to have decreased by less than 50% between 2000 and 2015. Nevertheless, this disease remains a significant challenge as malaria-related deaths still account for up to 11% and approx. 20% of total maternal and infant mortality, respectively (NPC, 2012).

Malaria in pregnancy is associated with severe symptoms in the mother primarily during the first and second pregnancies, as well as in the fetus and in the neonate. Medical complications include maternal hypoglycaemia, retarded fetal growth, placental malaria, abortion, fetal hypotrophy, premature birth, and low birth weight (Ugwu *et al.*, 2013, Takem & D'Alessandro, 2013, Mulumba *et al.*, 2003). Each year, approximately 10 million pregnant women are infected with malaria in Nigeria (NNMSP, 2017). The WHO recommends a series of interventions to prevent the adverse outcome of pregnancy due to malaria infection including the use of ITNs, IPTp with SP and effective case management among others. There has been an overall increase in access/ownership of ITNs in Nigeria but still less than one pregnant woman out of five receives the recommended 3 or more doses of preventive treatment during scheduled antenatal care (ANC) visits (NPC, 2012, NNMSP, 2017) Furthermore, only half of the pregnant women reported in 2015 attended monthly to the ANC visits as recommended from the second trimester of pregnancy (MIS, 2015, Figure 1.11).

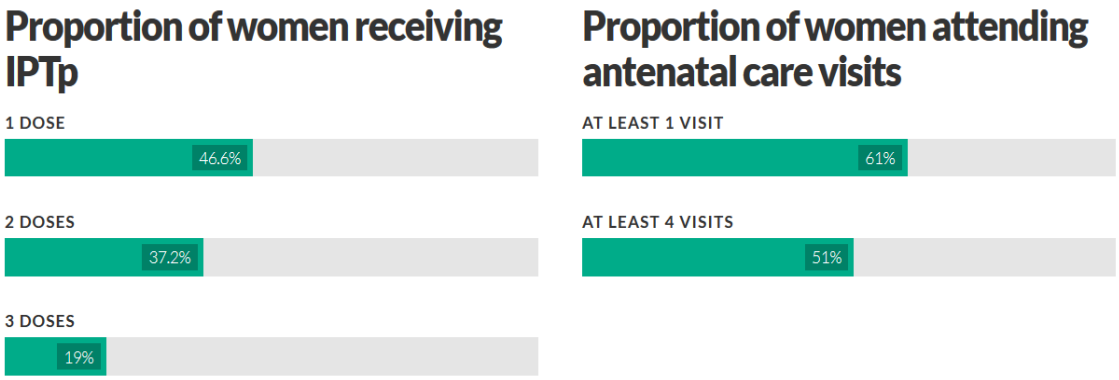

Figure 1.11: Proportions of pregnant women receiving IPTp and attending ANC in Nigeria, 2015. Figure adapted from MIS, 2015.

In 2018, a 5-year project called TIPTOP (acronym of Transforming Intermittent Preventive Treatment for Optimal Pregnancy) was started in 4 countries of sub-Saharan Africa, including Nigeria. This community-based program (now entering phase 2) aims to dramatically increase the number of pregnant women receiving IPTp (≥ 3 doses of SP) of 50% by distributing quality-assured SP in the communities and in the ANC clinics as well as to generate evidence to inform change in policy recommendations across sub-Saharan Africa (WHO, 2019b).

1.6.2. India

Although in the last decades India has achieved a major reduction in malaria prevalence, it still accounts for almost half of global vivax malaria cases (WHO, 2018). Despite treatment failure of chloroquine (CQ) in patients with *P. vivax* infection has been observed in several Asian countries including India (Rieckmann *et al.,* 1989, Baird *et al.*, 1991, Than *et al.*, 1995,

Baird *et al.*, 1996, Phan *et al.,* 2002, Barcus *et al.*, 2007, Baird *et al.*, 2009, Lee *et al.*, 2009), CQ is still the first-line treatment of *P. vivax* malaria in most endemic regions of SEA due to its clinical effectiveness and affordability, followed by the administration of primaquine.

The first Indian cases of CQ treatment failure were reported in 1995 in Bombay, and one year later in Uttar Pradesh (Garg *et al.,* 1995, Dua *et al.,* 1996). CQ-resistant *P. vivax* parasites have been subsequently detected in Bihar, in Odisha and in the western states of Delhi, Rajasthan, Maharashtra, and Gujarat (Adak *et al.,* 1998, Singh, 2000, Kshirsagar *et al.*, 2000, Yadav & Ghosh*,* 2002, Srivastava *et al.*, 2008, Wangdi *et al.,* 2016). *P. vivax* malaria is a serious concern in the Indian sub-continent, considering that 11% to 45% of patients hospitalized with *P. vivax* infection in this country reportedly develop severe malaria (Tanwar *et al.*, 2011, Limaye *et al*., 2012, Jain *et al.*, 2013, Kochar *et al.*, 2014).

Drug resistance in Indian *P. falciparum* isolates has emerged earlier than in *P. Vivax*. Already in the early 1970s, CQ-resistant *P. falciparum* parasites were observed in Northeast India (Sehgal *et al*., 1973). Sulfa–pyrimethamine combinations were soon recommended for treatment in CQ-resistant areas. In 2005, artesunate *plus* sulfadoxine–pyrimethamine (AS+SP) was introduced as second-line antimalarial drug for use in CQ treatment failures, and as first-line antimalarial treatment in areas with documented drug resistance. In 2007, AS+SP was selected for first-line treatment in high-risk districts and in 2010, it was introduced as the drug of choice for treatment of uncomplicated falciparum malaria in the whole country (NVBDCP, 2010).

The first case of resistance against pyrimethamine and sulfa-drugs in India dates back to 1979 in Assam, the state where also CQ resistance had emerged, and later on near the border to Myanmar (Das *et al.*, 1981, Mohapatra *et al.*, 2003). In 2013, seven north-eastern Indian states, *i.e*., Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland and Tipura, had to undergo a change in drug policies in view of the quick development of SP resistance (NVBDCP, 2013, Anvikar *et al.*, 2014). The current ACT in use in these federal states is arthemeter-lumefantrine (AL).

The efficacy of AS+SP has been threatened by the contemporary emergence of ART resistance in SEA. ART resistance was first reported in 2007 along the Thai-Cambodian border (Dondorp *et al.*, 2009, Noedl *et al.*, 2008). Recently, slow parasite clearance following ACT has been observed in western Thailand at the border with Myanmar, in Vietnam, and along the Myanmar-China border (White, 2011, Amaratunga *et al.*, 2012, Phyo *et al.,* 2012, Hien *et al.*, 2012).

The detection of *K13*-propeller variants of *P. falciparum* showing reduced sensitivity to ACTs in few Myanmar regions bordering India (Tun *et al.*, 2015) is extremely worrisome since the historical westward spread of drug-resistant strains could undermine long-term effectiveness of ACTs in India (Dondorp *et al.*, 2009).

Another important promoter of resistance development in both *P. falciparum* and *P. vivax* parasites is represented by the introduction of resistant malaria into non-immune populations such as refugees or migrants. Such a process increases the probability of resistance spread since parasites with low or moderate resistance would be cleared in semi-immune populations. In the same way, asymptomatic semi-immune migrants could promote the development of resistance in otherwise susceptible areas (Talisuna *et al.*, 2004).

1.6.3. Rwanda

Rwanda is an inland country of the Great Lakes region of eastern Africa and is bordered by Uganda, Burundi, Tanzania and the Democratic Republic of the Congo. With a population of approximately 12 million people distributed over 26,338 km^2, it represents the most densely populated country in continental Africa (Figure 1.12). Malaria has historically represented one of the major public health problem of the country. It caused a devastating and constant rise in child mortality rate between 1990-2000, with a peak of 300 /1,000 deaths of children under 5 years of age in 1994. Between 2001 and 2005, a stable malaria mortality rate of 10-14% has been registered (World Bank, 2019, Murray *et al.,* 2012).

In 2006, Rwanda has started a rapid scale-up in disease control, successfully improving health policies and interventions (WHO, 2006). AL has been adopted as first-line treatment for uncomplicated *P. falciparum* malaria and its wide distribution within the public-sector health facilities, was paralleled by a decline of more than 50% of malaria cases within one year from its introduction (Tambo *et al.*, 2012, Otten *et al.*, 2009).
Furthermore, the distribution of approx. 2 millions of long-lasting insecticide treated nets (LLINs) during a nationwide campaign targeting children <5 years in 2006-2007, have increased the percentage of population covered by LLINs to over 70% (WHO, 2009).

Figure 1.12: Geographical position of Rwanda (left and central picture) and on the right, **location of the district of Huye (red) in the southern province (pink)** where the current study was mainly performed (PMI, 2015).

In spite of such achievements, since 2012 the incidence of malaria in the country has steadily increased from 48 per 1,000 inhabitants in 2012 to over 400 per 1,000 in 2016. Between 2012 and 2016 the number of reported malaria cases has increased 8-fold from approx. 567,400 cases to over 4,790,000 (PMI, 2018). Such growth was particularly evident in the eastern and southern province of Rwanda where in recent years numbers of malaria cases have tripled (from 460,460 in 2013–2014 to 1.4 million in 2015–2016), and doubled (from 554,035 in 2013–2014 to over 1.1 million in 2015–2016), respectively.

Already in 2012, a study conducted by Zeile and co-workers, pointed out the prevalence in southern Rwanda of the *pfmdr1* haplotype N86-184F-D1246 indicative of intense AL pressure on the parasite population (Zeile *et al.,* 2012) (see section 1.5.1.1.). Lack of data concerning both *in vivo* and i*n vitro* partial ART resistance in this country are worrisome since an eventual onset of ART partial resistance would strongly undermine AL treatment outcome due to the potentially reduced efficacy of lumefantrine. In addition, Rwanda is geographically close to Uganda, the only African country where high *ex vivo* RSA survival rates in isolates from slow clearance infections (*i.e.*, infections with parasite clearance half life greater than 5 hours) have been observed (Ikeda *et al.*, 2018).

1.7. Objective of the work

The overall aim of this thesis is to provide information on the status of antimalarial drug resistance with respect to antimalarial drugs and geographical regions that suggest emerging drug resistance. This applies to ART resistance in Rwanda, AS+SP, AL and CQ resistance in India and SP resistance (IPTp) in Nigeria. For that, molecular markers of drug resistance are related to clinical significance, treatment outcome, and, for Rwanda, RSA results.

Specific aims

Study I To investigate for the first time the presence of *K13* polymorphisms in asymptomatic *Plasmodium falciparum* infections from southern Rwanda in samples collected between 2010 and 2015.

Study II To assess the degree of AS+SP resistance in *P. falciparum* isolates from Mangaluru, India, and to estimate the value of AL as an alternative.

Study III To determine *pvmdr1* gene diversity in vivax malaria patients from Mangaluru, searching for correlations between parasite clearance after CQ treatment and clinical, parasitological, and biochemical characteristics of malaria patients.

Study IV To determine the prevalence of *pfdhfr* and *pfdhps* polymorphisms found in pregnant women receiving intermittent preventive treatment of malaria with SP in Calabar, Nigeria,

Study V To evaluate *in vivo* parasite clearance by *ex vivo* assay on freshly collected *P. falciparum* isolates and revisit the potential correlations with previously and newly identified *K13*-propeller polymorphisms in Rwanda.

2. Materials and methods

2.1. Materials

Autoclave	Tuttnauer Systec 2540, Tuttnauer USA Co. Ltd., USA
Anaero-box	Thermo Fisher Scientific, Berlin, DE
P. falciparum incubator	Cytoperm 2, Heraeus GmbH, Hanau, DE
Pipetman, P2, P20, P200, P1000	Gilson, Middleton, WI, USA
Freezer (-80°C)	Heraeus GmbH, Hanau, DE
Freezer (-20°C)	Liebherr, Biberach, DE
Refridgerators	Liebherr, Biberach, DE
Magnetic stirrer	Janke & Kunkel Labortechnik, Berlin, DE
Microwave	AGE, Robert Bosch GmbH, Stuttgart, DE
PCR-Machine	T gradient Thermocycler, Biometra, Göttingen, DE T3 Thermoblock, Biometra, Göttingen, DE
pH-Meter inoLab ®	WTW GmbH, Weilheim, DE
Sterile work bench	ClassII Type A, The Baker Company, USA
Benchtop Centrifuge	Centrifuge 5417 R, Eppendorf, Hamburg, DE Centrifuge 5415 D, Eppendorf, Hamburg, DE
Thermo-mixers	Thermomixer 5436, Eppendorf, Hamburg, DE Thermomixer comfort, Eppendorf, Hamburg, DE
UV-Transluminator	SYNGENE, Synoptics Ltd., USA
Vortex	Vortex Genie 2, Scientific Industries, Dortumund, DE
Balance	Sartorius Portable, Sartorius Lab instruments GmbH & CO. KG, Göttingen, DE
Water bath	Julabo 7A, Julabo GmbH, Seelbach, DE
Centrifuge	Multifuge 1 S-R, Heraeus GmbH, Hanau, DE Multifuge 1.0 R, Heraeus GmbH, Hanau, DE
Microscope	LSM 510, Carl Zeiss Microscopy GmbH, Jena, DE
Class II A/B3 biosafety cabinet	HERA safe, Heraeus GmbH, Hanau, DE Thermo Fisher Scientific, Berlin, DE
Gel tray	Thermo Scientific Owl A2 Large Gel Systems, Thermo Fisher Scientific, Berlin, DE
Elecrophoresis system	Fisherbrand power supply, Berlin, DE

2.2. Disposables

Disposable gloves (Latex/Nitrile)	Hartmann, Heidenheim, DE
Film material	SONY GmbH, Berlin, DE
Cryovial (Plastic, Screw-cap)	Sarstedt, Nümbrecht, DE
Parafilm	American International Can., C, IL, USA
Plastic Pasteur pipette	Carl Roth, Karlsruhe, DE
PCR- tube	Corning Incorporation, Bodenheim, DE
Pipette tip 0,1-2,5µL, 2-20µL, 20µL-200µL, 100µL-1000µL	Eppendorf, Hamburg, DE
Culture flasks T25, T75	Sigma-Aldrich GmbH (TPP), Hamburg, DE
Serological Rotilabo ® tip 1 mL, 5 mL, 10 mL, 25 mL	Carl Roth, Karlsruhe, DE
Tube 1.5 mL, 2 mL, 15 mL	Sarstedt, Nümbrecht, DE
Tube Safe-Lock 2 mL	Eppendorf, Hamburg, DE
0.22 µm Rotilabo ® PVDF filter	Carl Roth, Karlsruhe, DE
0.2 µm Gas filter (PTFE)	Carl Roth, Karlsruhe, DE
50 mL-Syringe	Zinsser, Frankfurt, DE
Gas needle Sterican ®	Braun, Berlin, DE
3 MM Whatman paper	GE Healthcare Life Sciences, Berlin, DE
Filter paper	Schleicher & Schuell, Einbeck, DE
Immersion oil	Carl Zeiss Microscopy GmbH, Jena, DE
Microscope slides	Paul Marienfeld GmbH Woellerspfad, DE
Cotton pad	Braun, Berlin, DE
EDTA tube 1.5 mL	Becton, Dickinson & Co (BD). NJ, USA
Empty vacutainer 2.7 mL	Becton, Dickinson & Co (BD). NJ, USA
Glucose Analyzer	HemoCue Glucose 201 RT System, Hitado GmbH, Möhnesee, DE
Haemoglobin Analyzer	HemoCue Hb 201+, Hitado GmbH, Möhnesee, DE
Dipstick	Multistix 10 SG, Bayer HealthCare, Berlin, DE

2.3. Chemicals & Reagents

Ethanol 100%	Richter Chemie GmbH, Berlin, DE
Methanol 99.8%	Sigma-Aldrich GmbH, Hamburg, DE
RPMI 1640	Thermo Fisher Scientific (Gibco) Berlin, DE
Gentamycin (50mg/mL)	Sigma-Aldrich GmbH, Hamburg, DE
L-glutamine	Sigma-Aldrich GmbH, Hamburg, DE
HEPES buffer 1M	Sigma-Aldrich GmbH, Hamburg, DE
ACD solution	Sigma-Aldrich GmbH, Hamburg, DE
Sorbitol	Sigma-Aldrich GmbH, Hamburg, DE
Double-distilled water (ddH_2O)	Biochrom GmbH, Berlin, DE
Glycerol	Sigma-Aldrich GmbH, Hamburg, DE
Sodium Chloride (NaCl)	Sigma-Aldrich GmbH, Hamburg, DE
Hypoxantine	Sigma-Aldrich GmbH, Hamburg, DE
Sodium Bicarbonate ($NaHCO_3$)	Sigma-Aldrich GmbH, Hamburg, DE
RPMI 1640 w. Gln, w/o $NaHCO_3$	Thermo Fisher Scientific (Gibco) Berlin, DE
Albumax II powder	Thermo Fisher Scientific (Gibco) Berlin, DE
Dihydro-artemisinin (DHA)	Sigma-Aldrich GmbH, Hamburg, DE
Giemsa	Sigma-Aldrich GmbH, Hamburg, DE

2.3.1. Buffers and Solutions

Complete medium for culturing (CMC)	500 mL liquid RPMI 1640 100 ng/mL gentamycin 2 mM L-glutamine 25 mM HEPES buffer 25 mM $NaHCO_3$ 0.5% Albumax II solution
CMC for field study*	RPMI1640 medium w/ Gln, w/o $NaHCO_3$ 25 mM HEPES buffer 0.1 M hypoxanthine NaOH (as required to adjust pH to 7.2-7.4) 25 mM $NaHCO_3$ 100 ng/mL gentamycin 1% Albumax II powder

Washing medium	500 mL RPMI 1640 50 ng/mL gentamycin
Freezing solution*	3% sorbitol 0.65% NaCl 28% glycerol in double-distilled water (ddH_20)
10x TBE buffer	890 mM Tris base 890 mM boric acid 20 mM EDTA, pH=8
Thawing solution 1*	12% NaCl in ddH_20
Thawing solution 2*	1.6% NaCl in ddH_20
10x Albumax II solution*	50 ng/mL gentamycin 0.1 M hypoxanthine 25 mM HEPES buffer 25 mM $NaHCO_3$ 0.5% Albumax II powder in ddH_20
DHA stock solution*	1 mg/mL DHA in dimethyil sulfoxide (DMSO)
DHA aliquots ready-to-use*	200 µg/mL DHA in DMSO
Giemsa staining	5-10% Giemsa in ddH_20

*solutions were prepared on sterile bench and filter-sterilized in the safety cabinet.

2.3.2. Commercial Kits

QIAamp DNA Blood Mini Kit (250)	QIAGEN, Hilden, DE
DNA Polymerase Kit: HotStarTaq DNA Polymerase	QIAGEN, Hilden, DE
BinaxNOW® Malaria Test (RDT Kit)	Alere Scarborough Inc., ME, USA
SD Bioline Malaria Ag Pf (HRP2/pLDH) RDT Kit	Codex Pharma Ltd., Nigeria

2.3.3. Markers

marker 1 kb-DNA ladder	New England BioLabs (NEB) GmbH, Berlin, DE
marker 1 kb plus-DNA ladder	Thermo Fisher Scientific, Berlin, DE
marker 100 bp-DNA ladder	Thermo Fisher Scientific, Berlin, DE

2.3.4. Oligonucleotides

Primers were purchased from Eurofins Genomics, Berlin, DE or from Tib MOLBIOL Berlin, DE. Sequencing was performed by GATC, Konstanz, DE (belonging to Eurofins Genomics) and is described in more detail in section 2.7.6.2. PCR-RFLP technique used to investigate polymorphisms in *pfdhfr* and *pfdhps* in samples from Calabar, Nigeria is described in section 2.7.6.5.

Primer name	**Sequence 5' – 3'**	**Source**
rPLU6 (F)	TTAAAATTGTTGCAGTTAAAACG	Snounou *et al.*, 1993
rPLU6 (R)	CCTGTTGTTGCCTTAAACTTC	Snounou *et al.*, 1993
rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	Snounou *et al.*, 1993
rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	Snounou *et al.*, 1993
rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC	Snounou *et al.*, 1993
rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA	Snounou *et al.*, 1993
rOVA1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	Snounou *et al.*, 1993
rOVA2	GGAAAAGGACACATTAATTGTATCCTAGTG	Snounou *et al.*, 1993
rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	Snounou *et al.*, 1993
rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	Snounou *et al.*, 1993
K13 out F	GGGAATCTGGTGGTAACAGC	Ariey *et al.*, 2014
K13 out R	CGGAGTGACCAAATCTGGGA	Ariey *et al.*, 2014
K13 in F	GCCTTGTTGAAAGAAGCAGA	Ariey *et al.*, 2014
K13 in R	GCCAAGCTGCCATTCATTTG	Ariey *et al.*, 2014

Pvmdr1_F1	TTGAACAAGAAGGGGACGTT	Lu *et al.,* 2011
Pvmdr1_R1	CTTATATACGCCGTCCTGCAC	Lu *et al.,* 2011
Pvmdr1_F2	CAGCCTGAAAGATTTAGAAGCCTT	Lu *et al.,* 2011
Pvmdr1_R2	CATCCACGTCCACAGTGGAAC	Lu *et al.,* 2011
Pvmdr1_F3	GGATAGTCATGCCCCAGGATTG	Lu *et al.,* 2011
Pvmdr1_R3	CATCAACTTCCCGGCGTAGC	Lu *et al.,* 2011
Pvmdr1_F7	GATGAGCCTGCTGATGCGATTCTAC	Lu *et al.,* 2011
Pvmdr1_R5	ATATACGCCGTCCTGCACCGAG	Lu *et al.,* 2011
Pfdhfr_M1	TTTATGATGGAACAAGTCTGC	Duraisingh *et al.,* 1998
Pfdhfr_M5	AGTATATACATCGCTAACAGA'	Duraisingh *et al.,* 1998
Pfdhfr_M3	TTTATGATGGAACAAGTCTGCGACGTT	Duraisingh *et al.,* 1998
Pfdhfr_F/	AAATTCTTGATAAACAAC GGA ACCTttTA'	Duraisingh *et al.,* 1998
Pfdhfr_F	GAAATGTAATTCCCTAGATATGgAATATT	Duraisingh *et al.,* 1998
Pfdhfr_M4	TTAATTTCCCAAGTAAAACTATTAGAg CTTC	Duraisingh *et al.,* 1998
Pfdhps_R2	AACCTAAACGTGCTGTTCAA	Duraisingh *et al.,* 1998
Pfdhps_R/	AATTGTGTGATTTGTCCACAA	Duraisingh *et al.,* 1998
Pfdhps_K	TGCTAGTGTTATAGATATAGGatGAGcATC	Duraisingh *et al.,* 1998
Pfdhps_K/	CTATAACGAGGTATTgCATTTAATgCAAGAA	Duraisingh *et al.,* 1998
Pfdhps_J	TGCTAGTGTTATAGATATAGGTGGAGAAagC	Duraisingh *et al.,* 1998
Pfdhps_L	ATAGGATACTATTTGATATTGGAccAGGATTcG	Duraisingh *et al.,* 1998
Pfdhps_L/	TATTACAACATTTTGATCATTCgcGCAAccGG	Duraisingh *et al.,* 1998
Pfmdr1_A1	TGTTGAAAGATGGGTAAAGAGCAGAAAGAG	Duraisingh *et al.,* 2000
Pfmdr1_A3	TACTTTCTTATTACATATGACACCACA AACA	Duraisingh *et al.,* 2000
Pfmdr1_A2	GTCAAACGTGCATTTTTTATTAATGACCAttTA	Duraisingh *et al.,* 2000
Pfmdr1_A4	AAAGATGGTAACCTCAGTATCAAAGAAGAG	Duraisingh *et al.,* 2000
Pfmdr1_O1	AGAAGATTATTTCTGTAATTTGATACAAAAAGC	Duraisingh *et al.,* 2000

Pfmdr1_O2	ATGATTCGATAAATTCATCTATAGCAGCAA	Duraisingh *et al.,* 2000
Pfmdr1_1034f	AGAATTATTGTAAATGCAGCTTTATGG GGAcTC	Duraisingh *et al.,* 2000
Pfmdr1_1042r	AATGGATAATATTTCTCAAATGATAAcTTaGCA	Duraisingh *et al.,* 2000
Pfmdr1_1246f	ATGATCACATTATATTAAAAAATGATATGACAAAT	Duraisingh *et al.,* 2000

2.4. *Plasmodium* strains

Plasmodium falciparum laboratory strains NF54, HB3 and Dd2 stored at -80°C and available at the Institute of Tropical Medicine (ITM) of the Charité-Universitätsmedizin Berlin have been used for the preliminary assessments of short-term and long-term culture systems including optimization of continuous culturing conditions.

Furthermore, field isolates obtained from malaria patients attending Charité in the last years returning from Cambodia, Tanzania, Nigeria and South Africa, respectively, have been used to establish long-term *in vitro* cultures of *P. falciparum* as well as to assess culture adaptation for drug pre-testing assays.

One parasite isolate collected from a seven years old patient attending ITM with severe malaria and hyperparasitaemia (25%) was considered of particular interest. In this patient, parasitaemia declined rapidly during the first five days of treatment but then persisted at around 5% for five additional days, despite daily intravenous artesunate administration. Low levels of parasitaemia persisted up to Day-22 after initiation of treatment. At light microscopy, parasites appeared pyknotic and molecular analysis of *K13*-propeller on parasites obtained on Day-2, -4, and -5 of treatment revealed no point mutation associated with ART resistance (Bélard *et al.*, 2019, *Manuscript under review*).

2.5. Gas mixture

Tri-gas atmosphere: 5% O_2, 5% CO_2, 90% N_2 (Linde GmbH, Berlin, DE) has been used to flush short-term and long-term cultures obtained from both frozen laboratory strains and patient samples. The candle-jar technique of Trager–Jensen has been used during the Rwandan field study to perform ring-stage survival assays (RSAs) (Witkowski *et al.*, 2013).

2.6. Software & Database

PlasmoDB	http://plasmodb.org/plasmo/
NCBI	www.ncbi.nlm.nih.gov/pubmed/
Bioedit v.7.0	Ibis Therapeutics, Carlsbad, CA, USA
SnapGene v.3.1	GSL Biotech LLC., Chicago, USA
SPSS v.22	IBM Corp., Armonk, NY, USA
Statview v.5.0	SAS Institute Inc., Cary, NC, USA
Microsoft Office 2017	Microsoft Corp., Washington, DC, USA

2.7. Methods

2.7.1. Study sites and selection of participants

In this section, a brief description of the study sites is provided. At these locations, patient enrolment and clinical examination as well as sample and data collection were performed. DNA extraction and molecular analysis to detect polymorphisms in markers of resistance have been all performed at the ITM of Charité-Universitätsmedizin Berlin, in Germany.

2.7.1.1. Huye district, southern province of Rwanda

Under an administrative point of view, Rwanda consists of Kigali City and 4 provinces, divided into 30 districts, which include several sub-districts and ca. 15,000 villages. Sample collection for Study I and V (section 1.7.) was carried out in the Huye district of the southern province, mostly in Huye sub-district (PMI, 2016, Figure 2.1a).

Huye is an epidemic-prone district bordered to the north by several malaria endemic areas within the southern province such as Muhanga and Nyanza, and the high-burden district of Ruhango. Moreover, since 2015, Huye district is highly exposed on the south side to the influx of refugees coming from neighbouring Burundi.

Blood specimens and patient data of Study I were collected in 3 time lapses. The first collection was performed in January-March 2010 as part of a monitoring study on the prevalence of malaria and soil-transmitted helminths in children under 5 years of age. Children from the communities were recruited from each twenty-five households randomly selected among 24 villages of the rural Huye sector as described elsewhere (Figure 2.1b, Gahutu *et al.*, 2011).

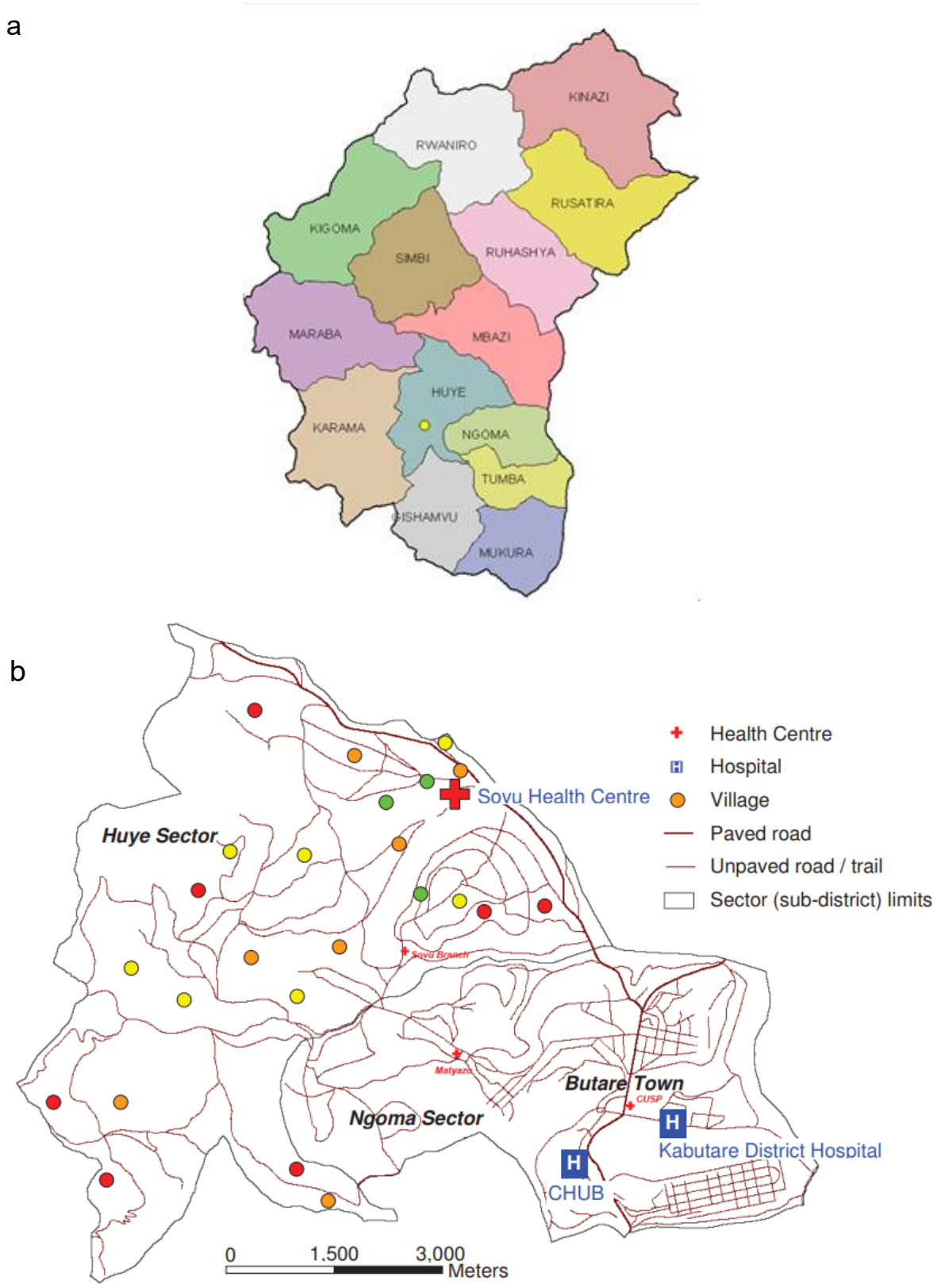

Figure 2.1: (**a**) **Huye district accounts for 14 sub-districts, including Huye (light-blue)**. The main location of Study I and V within Huye sub-district is highlighted by a yellow dot (PMI, 2016). (**b**) **Catchment area of Study I in Huye sub-district in 2010**. The prevalence of *P. falciparum* infection among recruited children is marked by colour: green, <5%; yellow, 5 ≤ 10%; orange, 10 ≤ 20%; red, >20% (Gahutu *et al*., 2011).

Chosen households were visited by community health workers to recruit one child per family, who was asked to visit Sovu Health Centre (or a non-permanently staffed branch) on the following day. Furthermore, paediatric patients ≤5 years of age attending Sovu Health Centre and Kabutare District Hospital (KDH), respectively, were enrolled in the study. The second sample collection was carried out in October 2014 on community children attending primary school as part of a study on the effectiveness of routine de-worming. Twelve sub-districts of Huye district (all sub-districts excluding Simbi and Ruwaniro, Figure 2.1a) were included in the study. One primary school of approx. 500–1000 children was randomly selected for each sub-district. At each school, 150 children aged 6–10 years were randomly chosen to participate to the study as described elsewhere (Sifft *et al.,* 2016). The third sample collection was carried out on an additional sub-set of 12 schools in the Huye district in October 2015 following the same protocols of the collection performed in 2014 (Krücken *et al.*, 2017).

For Study V, more than 200 uncomplicated malaria out-patients were enrolled at the primary Health Centres of Sovu and Rukira (sub-branch of Sovu Health Centre) between March and May 2018. A few cases of severe malaria were additionally recruited at KDH for molecular analysis only. The average age of the recruited patients was 23 years (range: 10-32) and 55% were males.

2.7.1.2. Mangaluru, Karnataka, south-western India

Mangaluru (also called Mangalore) is a harbour city of 485,000 inhabitants located at the Arabian Sea in the state of Karnataka, south-western India. Studies II and III were performed at the malaria diagnostic unit of Wenlock hospital. Wenlock hospital is the largest governmental hospital in Mangaluru (900 beds) offering treatment particularly for the economically deprived part of the population. In addition, several private hospitals provide health services in the absence of primary health care facilities in this urban setting.

In 2014, Wenlock Hospital reported 6,767 malaria cases, 80.1% being *P. vivax* mono-infections. Patients attending the out-patient department (OPD) of the hospital's malaria diagnostic unit between June and December 2015 were enrolled in Study II and III. Upon recruitment, venous blood was collected from the patients into EDTA tubes and additionally spotted onto Whatman filter paper. Among the 909 malaria patients recruited, the vast majority was male (93%), adult (median, 26 years), and of low socio-economic status. Importantly, approx. 50% of them were migrants from beyond the local Karnataka state, mostly from northern and north-eastern Indian states. Clinical and socio-economic conditions of the enrolled patients have been better described elsewhere (Gai *et al.,* 2018).

2.7.1.3. Calabar, Cross River, southern Nigeria

Study IV was carried out at the antenatal (ANC) clinic of the General Hospital of Calabar, in the state of Cross River, in southeast Nigeria alongside a clinical trial. The General Hospital is the largest government-owned, secondary health facility of the city and provides health assistance to most of Calabar inhabitants. Since August 2009, pregnant women and children under five years old receive free medical care as part of a funded welfare program by the Cross River state government. The average number of annual ANC clinic attendances and deliveries at the hospital are 16,550 and 3,100, respectively (Esu *et al.*, 2018b).

Malaria transmission in this area is intense and perennial due to the favourable tropical-humid climate (15-30°C with mangrove swamp forest as predominant vegetation). In the Population and Housing Census of 2006, 1,471,967 males and 1,421,021 females were living in Cross River with an annual growth rate of 2.9%. The projected population for 2015 was 3,783,085 (WHO, 2016, NPC, 2012).

Intermittent preventive treatment in pregnancy (IPTp) with SP is recommended in all African areas with moderate and high transmission of malaria, including Nigeria (section 1.6.1.). A total of 460 women in their second trimester of pregnancy with asymptomatic malaria were recruited in the study. These pregnant women were attending their first ANC visit and had not received any dose of IPTp-SP at the time of enrolment. Upon recruitment, finger-prick blood samples were collected to determine Hb levels, for the preparation of thick blood smears for malaria parasite detection and counts and for the preparation of dried blood spots on filter paper. All enrolled women received a long-lasting insecticide treated net (LLIN) and a daily supplement of folic acid (4 mg) and ferrous sulphate (200 mg) tablets in accordance to national policy. Furthermore, they were asked to return at week 24 (6^{th} month of pregnancy), 32 (8^{th} month) and 36 (9^{th} month) of gestation for ANC follow-up visits. At delivery, birth weight and possible miscarriages, neonatal deaths or congenital abnormalities in the new-borns were recorded by midwives. Enrolled women showing fever or symptoms related to malaria were screened for malaria infections and promptly treated with quinine (30 mg/kg daily for 5 days) if positive.

2.7.2. Patient registration and samples collection

Patient enrolment and sample collection were carefully planned ahead and performed in an appropriate location. Local trained staff was provided with written, readily available SOPs. For blood sampling, a system of identification and tracking of the collected blood was established to ensure that the sample would be correctly matched with patient‘s data. When the study was

carried out at different locations blood products were placed in sealed boxes for safe transportation. The Nigerian field study (Study IV) was carried out alongside a clinical trial that has been detailed elsewhere (registration No.: PACTR201308000543272) while studies conducted in India and Rwanda (Study I, II, III and V) followed similar collection procedures. Patient enrolment and blood specimen collection of Rwandan Study V will be here described in more details as example.

Patient attending the out-patient department of Sovu or Rukira Health Centre (Study V) were registered and pre-screened by a trained nurse. Pre-screening included measuring body temperature and assessing *Plasmodium* infection by rapid diagnostic test (RDT). RDTs are plastic or paper cassettes containing a strip made of nitro-cellulose with dye-labelled antibodies (Figure 2.2). If after blood (and buffer) application the antibody binds to a parasite antigen, the resultant complex is captured on the strip forming a visible line (T-test line) in the results window. A control line (C-control line) gives information on the integrity of the antibody-dye conjugate.

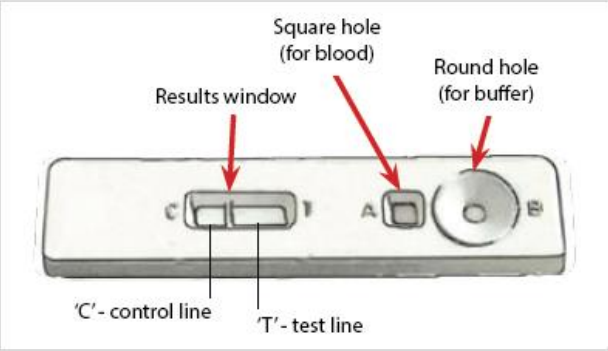

Figure 2.2: Rapid diagnostic test (RDT) cassette. A drop of blood and of buffer are added to the square hole and the round hole, respectively. Buffer carries the blood along the length of the RDT. Results are visualized on the result window (CDC, 2018).

Patients with fever at the time of registration (*i.e.,* axillary temperature ≥37.5°C) or history of fever in the past 48 hours that resulted positive for *Plasmodium* infection by RDT were considered suitable for the enrolment in the study. Enrolment was performed by providing written consent to participate to the study. Consent form as well as adequate information to allow an informed decision were given in Kinyarwanda (local language) to the patient or to his/her guardian in case of children. Participation in the study was voluntary; subjects were free to withdraw from the study at any time, and this did not affect their access to or quality of care. Information obtained from all subjects were treated as confidential.

Anthropometric measurements including body weight, blood pressure, height and mid-upper arm circumference (MUAC) were taken from the participants that were then introduced to the phlebotomist for venous blood collection (section 2.7.4.) (Figure 2.3).

Figure 2.3: (**a**) **Sovu Heath Centre** was the main site of out-patients recruitment and blood sampling (Photo by: Costanza Tacoli). (**b**) **Sovu Health Centre rooms allocation.** Enrolled patients had an established trail to follow including registration in room 1, anthropometric measurements in room 2, blood sampling and laboratory assessment of parasitaemia in room 3 and 4, clinical examination and socio-economic data collection in room 5 and 6.

Two blood specimens were collected in EDTA tubes and in acid citrate dextrose (ACD) pre-filled vacutainers for long-term storage and *ex vivo* ring-stage survival assay (RSA), respectively.

Few drops of EDTA sample were used at the laboratory of the health facility to roughly assess *Plasmodium* parasitaemia using the “plus system” method on a thick blood smear. This counting method relies on fields count rather than count *per* white blood cells (WBCs).

Although it is less precise since the variation in the thickness of the film may result in a false variation of parasite count, it is routinely used in Rwandan health centres for its speed and convenience. “Plus system” criteria are the following:

+ = 1–10 parasites *per* 100 thick fields
++ = 11-100 parasites *per* 100 thick fields
+++ = 1–10 parasites *per* one thick field
++++ = >10 parasites *per* one thick field

EDTA blood specimen was further used for quantitative determination of haemoglobin (Hb) and glucose in whole blood using HemoCue devices (HemoCue Glucose 201 RT System and HemoCue Hb 201+ from Hitado GmbH, Möhnesee, DE) and to prepare filter paper blood spots, stored at the local laboratory as back up.

After blood collection, patients were directed to the doctor for history-taking and physical examination (section 2.7.4.). Patients with malaria were treated with artemether-lumefantrine, and other diseases were treated according to Rwanda health authority guidelines (MoH, 2018). Patients took their first dose of ACT in the presence of a nurse/co-worker and patient was kept under observation for 30 minutes to report a case of vomiting. Before dismission, the patient was requested to return to the health centre after 3 days of treatment to assess residual parasitaemia.

ACD tubes filled with full blood as well as the remnants of EDTA tube specimen were transported by one member of the staff to the University Hospital in Butare (CHUB) (Figure 2.4). EDTA tubes were used to prepare whole blood aliquots for future DNA extraction as well as for plasma isolation. Since EDTA anticoagulant affects parasite survival rates, ACD pre-filled tubes were used for the cryopreservation of infected erythrocytes’ pellet and for the *ex vivo* RSA processing. *Plasmodium* species and accurate parasitaemia were assessed by two different microscopists on Giemsa-stained thick and thin blood smears at the CHUB.

Figure 2.4: University Teaching Hospital of Butare, *Centre Hospitalier Universitaire de Butare* (CHUB). Main site of laboratory assessment and sample processing in Study V (Photo by: Costanza Tacoli).

2.7.3. Ethical consideration

Informed consent forms were provided by a selected field team in the local language. All methods were performed in accordance with the relevant guidelines and regulations. Inclusion in the study/trial proceeded upon signed acceptance of its terms and conditions by the patient or his/her legal guardian as appropriate prior to the research.

Study I & V were approved by the Rwandan National Ethics Committee (RNEC).

Study II & III protocols were reviewed and approved by the Institutional Ethics Committee of Kasturba Medical College, Mangaluru, Manipal University, and permission to conduct the study was granted by the Directorate of Health and Family Welfare Services, Government of Karnataka, India.

Study IV was approved by two ethics committees: Cross River Health Research Ethics Committee, Calabar, Nigeria, and the Ethics Board of the Medical Centre of Ludwig-Maximilians-University (LMU), Munich, Germany.

2.7.4. Patient clinical examination and data collection

Upon recruitment, patients were interviewed using preformed questionnaires on socio-economic parameters including education, occupation, household characteristics and malaria-related behavior. Medical history was taken and clinical examination by a physician was performed. Examination included determination of weight, height, MUAC, Hb concentration, body mass index (BMI) calculated as kg/m^2 and of fever at the time of enrollment, defined as axillary temperature over or equal to 37.5°C. Hb and glucose levels were measured by a HemoCue photometer.

Venous blood was collected into EDTA tubes for parasitaemia assessment. Malaria parasites were counted *per* 200 WBCs on Giemsa-stained thick blood films by two trained microscopists independently. Briefly, 200 leucocytes were counted in approx. 100 fields (0.25 µL of blood). Asexual parasites were counted in relation to a predetermined number of estimated WBCs present in an individual (8000/ mm^3). This amount was taken as standard to assess parasites/µL of blood in accordance to the following formulae:
(No. of parasites/ No. of WBCs counted) x 8000 = No. of parasites/µL

In the Rwandan Study V, to avoid performing *ex vivo* RSA on specimens with parasitemia lower than 0.1% and higher than 1% (according to RSA procedure, section 2.7.5.6.), the number of iRBCs was assessed considering a WBC count of 5,000 and 10,000/mm^3, *i.e.*, the min. and max. for children older than 2 years of age up to adults.

Parasite species were assessed on Gimsa-stained thin blood smears by light microscopy. Following DNA extraction, *Plasmodium* species was ascertained by nested polymerase chain reaction (PCR) assay as described elsewhere (Snounou *et al.*, 1993) (section 2.7.6.2.).

Additional clinical parameters were evaluated for the Indian Study II and III such as WBCs and thrombocyte counts (Coulter principle) as well as concentrations of creatinine (Jaffé reaction), total bilirubin (DPD method), and direct bilirubin (Jendrassik-Grof method). Abnormal values in these parameters were defined as in Table 2.1 (Sullivan *et al.,* 2008). Severe malaria was defined based on the current WHO definition. In particular, hypotension (systolic blood pressure <80 mmHg in adults and <70 mmHg in children) and confusion in adult patients with a Glasgow coma score >11 were considered indicative of severe malaria irrespective of the absent assessment of capillary refill or impaired perfusion. Other important signs of severe malaria were renal impairment (plasma creatinine >3 mg/dL, or urea >20 mM), jaundice (plasma bilirubin >3 mg/dL *plus* parasitaemia >100,000/µL) and severe anaemia (Hb <5 g/dL in children, or Hb <7 g/dL in adults *plus* parasitaemia >10,000/µL; no parasite density threshold for vivax malaria).

Parameter	Value
anaemia (Hb)	<11g/dL (< 5 years) <11.5 g/dL (5<12 years), <12 g/dL (12<15 years, or females ≥15 years) <13 g/dL (males ≥15 years)
leukocytosis	>10,000 WBCs/µL
thrombocytopenia	<150,000/µL
increased creatinine	>1.4 unit/µL
increased total bilirubin	>1.2 mg/dL
increased direct bilirubin	>0.2 mg/dL

Table 2.1: Criteria used to identify abnormal values of Hb, WBCs, creatinine, platelets and bilirubin. These parameters were assessed in Indian malaria out-patients attending Wenlock Hospital during the rainy season in 2015.

Acidosis and hypoglycaemia were not routinely assessed. Clinical and socio-economic data collected from Mangalurean patients enrolled in Study II and III are described elsewhere (Gai *et al.*, 2018). Children of 5 or less years of age from the Huye district enrolled in Study I were further screened for intestinal parasites infections by microscopical stool examination, and for urinary tract infection (UTIs) by dipstick (Multistix 10 SG, Bayer HealthCare, Berlin, DE) as described elsewhere (Gahutu *et al.*, 2011).

2.7.5. Methods of *Plasmodium falciparum* culturing

2.7.5.1. Establishment of *in vitro P. falciparum* culture

The *in vitro* adaptation and culturing of parasite isolates collected from in- and out-patients with *P. falciparum* malaria attending the Institute of Tropical Medicine (ITM) of Charité-Universitätsmedizin Berlin was performed to better assess parasite viability, physiology and behaviour and to evaluate drug susceptibility.

Briefly, blood samples obtained from patients at the ITM were collected in EDTA tubes and processed for immediate cultivation within the following 12 hours. Anti-coagulated blood samples were centrifuged for 8 min. at 600 × *g* and the supernatant discarded. Samples were washed 3 times in prewarmed (37°C) washing medium (centrifuged for 8 min. at 600 × *g*). The buffy coat was carefully removed under a sterile hood after each centrifugation. Infected RBCs

pellet was resuspended in 5 mL prewarmed complete medium for culturing (CMC) and placed in a 25 cm^2 (T25) culture flask. Uninfected erythrocytes (uRBCs) obtained from human blood type A^+ donors were added to reach a final haematocrit of ~4%. The flask was flushed using a 0.2 µm filter unit and sterile needle with tri-gas mixture for ~30 s at 2 bar pressure and incubated at 37 °C for 24 h.

After incubation, the culture flask was removed from the incubator and a tiny amount of RBCs pellet was taken up with a sterile Pasteur pipette to make a Giemsa-stained thin smear (section 2.7.5.4.). The culture was then sub-cultivated to keep a final parasitaemia of 1 to 2%. Sub-cultivation was achieved by addition of new (prewarmed) culture medium and uRBCs to the flask and return to incubation at 37°C after flushing with tri-gas mixture. When required, the culture was sub-cultivated in 75 cm^2 flasks (T75, gas flushing time of ~90 s). Bigger splits of the culture (over 50%) were supported by further addition of uRBCs to keep haematocrit. Washed uninfected RBCs for sub-culture were kept at 4°C for 24 h before first usage to discourage any remaining leukocytes and used within the following two weeks.

Parasite growth and culturing conditions of both culture-adapted field strains and laboratory strains were tested and adjusted for use in the Rwandan field Study V to optimise *ex vivo* RSA procedure. This included a protocol change to cope with local settings and equipment, *e.g.*, use of a candle jar instead of a CO_2-floated incubator in the absence of a tri-gas atmosphere (Witkoswki *et al.,* 2013) as well as the preparation of CMC from powder instead of liquid base and the manual sterilization (by syringe) of most solutions.

2.7.5.2. Cryopreservation

Cryopreservation was performed at ITM on patient isolates after culture-adaptation (2 weeks of continuous culturing) and on laboratory strains (HB3, Dd2, NF54) for back-ups. In addition, infected RBCs (iRBCs) pellet of Rwandan *P.falciparum* isolates from Study V were cryopreserved for long-term storage and shipment.

Ideal cultures to freeze had high parasitaemia (5-15%) and were previously sorbitol-synchronized (section 2.7.5.5.) to have ring-stage parasites on the day of cryopreservation. The culture to freeze was centrifuged for 8 min. at 600 × *g* and supernatant was removed. An equal volume of ring-stage parasitized packed RBCs and freezing solution (see section 2.3.1) were mixed together taking into account cryovials capacity (max 1.8 mL). The mixture was then immediately poured into labelled cryovials and placed at -80°C.

2.7.5.3. Thawing

During parasite thawing, freezing solution is replaced by a highly salted solution (12% NaCl). Salt concentration is then slowly reduced to culturing conditions. This change in osmotic pressure must be carried out very carefully to allow cell adaptation to each solution and avoid RBCs lysis.

On the day of use, cryopreserved samples stored at -80°C were removed from cold storage and let thaw down at room temperature (RT) or in a 37°C water bath. Immediately after thawing, the iRBCs suspension was transferred to a 50 mL tube and an equal volume (ratio 1:1, approx. 1 mL) of thawing solution 1 (12% NaCl) was added dropwise and let stay at RT for 5 minutes. Approx. 9 mL (ratio 1:10) of thawing solution 2 (1.6% NaCl) were added at a rate of 1 drop per second to avoid lyses of erythrocytes. The suspension was centrifuged, the supernatant was discarded and prewarmed washing medium was added. The erythrocytes pellet was washed a total of 3 times in prewarmed washing medium before addition of uRBCs and CMC (see section 2.7.5.1.).

2.7.5.4. Determination of parasitaemia

Parasite growth in culture was daily assessed by iRBCs count on Giemsa-stained thin blood smears. A drop of sedimented erythrocytes was taken up from the bottom of the culture flask using a Pasteur pipette, placed onto the surface of a clean glass slide and spread evenly. The smear was dried and fixed for 1 min. in 100% methanol. The fixed cells were then stained in 5-10% Giemsa solution for 20 min. Slides were rinsed and air dried after staining and examined on a light microscope using a 100x objective under oil immersion. A count of 1,000 RBCs was considered reliable for parasitaemia assessment.

2.7.5.5. Synchronization of *P. falciparum* culture with sorbitol

Prolonged sub-culturing of untreated *P. falciparum* leads to asynchronous parasite growth *in vitro*. For experimental design, a synchronized parasite population was needed. Synchronization was performed when ring-stages where prevalently detected in thin blood smears.

Briefly, the culture was centrifuged (5-8 min. at 600 × *g*), CMC replaced by 5% sorbitol solution, and incubated for 10 min. at 37°C or left at RT for approx. 20 min. After incubation, sorbitol solution was removed by centrifugation and replaced by new complete medium. The culture was brought back to normal culturing conditions.

Since sorbitol processing leads to partial parasite death, this treatment depletes the culture of all mature, metabolically active blood stages, *i.e.*, trophozoites and schizonts. Ring-stages are not affected by the treatment. To obtain complete synchronization, the procedure was repeated in two to three consecutive replication cycles.

2.7.5.6. Ring-stage survival assay (RSA)

The ring-stage survival assay (RSA) is a drug susceptibility test developed by Witkowski *et al.* in 2013 as a new protocol to identify isolates with a slow clearing rate phenotype *in vitro* thus that can survive pharmacologically relevant doses of ART (Dondorp *et al*., 2009, Witkowski *et al*., 2013). This assay exists in two forms: i) the *in vitro* RSA$^{0\text{-}3h}$ is performed on culture-adapted parasite lines and is useful in elucidating the molecular basis of ART resistance by for instance experimentally validate candidate molecular markers; and ii) the *ex vivo* RSA performed on uncultured parasite isolates collected directly from malaria patients in the field which is recommended for mapping the geographical emergence and spread of ART-resistant parasites in real-time, thus providing actionable information for national malaria control programmes (Witkowski, *et al.*, 2013, Ménard *et al.*, 2015). The latter has been used to assess ART resistance emergence in Rwanda in 2018 (Study V).

In RSAs, parasites are exposed to 700 nM (nmol/L) of dihydro-artemisinin (DHA) for 6 hours to mimic clinical concentrations of the drug *in vivo* (Figure 2.5).

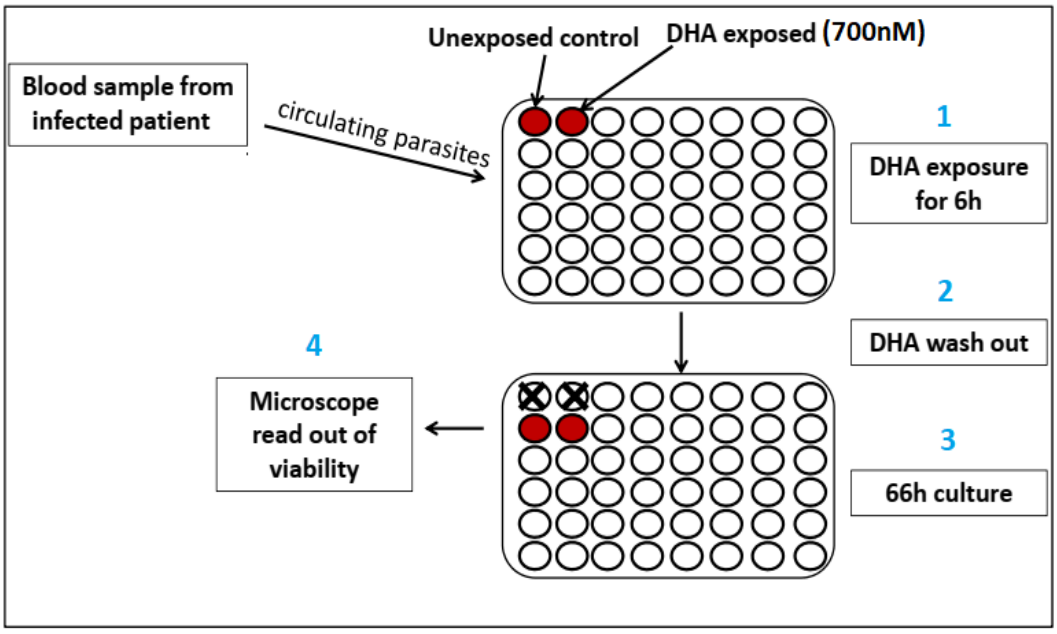

Figure 2.5: steps (1-4) of *ex vivo* ring-stage survival assay (RSA). Infected RBCs pellet are first pulse-exposed to DHA for 6 hours, then let recover for 66 hours after drug removal. Parasite viability is assessed by light microscopy. Figure adapted from ISC & NIH, 2015.

The percentage of viable parasites is then assessed after a recovery phase by comparison between drug-exposed parasites and non-exposed controls. Importantly, only clinical isolates with a parasitaemia between ≥0.1% and <1% collected from patients with *P. falciparum* mono-infection are considered suitable for the *ex vivo* RSA.

Briefly, aliquots of DHA solution were prepared in advance and stored at -20°C until use. DHA powder (Sigma) was resuspended in dimethyl sulfoxide (DMSO) to obtain a 1 mg/mL stock solution. The DHA stock solution was then diluted in 5-fold volume of DMSO and 50 µL aliquots "ready-to-use" (Fc= 200 µg/mL) were prepared in sterile 1.5 mL microcentrifuge tubes and stored at -80°C until use.

Ex vivo RSAs were performed on 2.7 mL of venous blood directly collected into acid-citrate-dextrose (ACD) vacutainers before the first treatment dose and processed within 24 h as described elsewhere (Witkowski *et al.*, 2013). Briefly, plasma was removed, and the RBCs pellet washed three times with complete RPMI-1640 medium (CMC), *i.e.*, RPMI-1640 medium supplemented with gentamicin solution at 0.01 mg/mL, 25 mM HEPES buffer, 25 mM $NaHCO_3$, and 0.5% Albumax II solution. Washed infected RBCs were exposed to 700 nM of DHA (drug-exposed) or to 0.1% DMSO (unexposed control) for 6 h and then washed 3 times in RPMI-1640 to remove the drug, re-suspended in CMC and maintained at 37°C in 5% CO_2 using a candle jar (Figure 2.5).

Survival rates were assessed independently by two microscopists by counting on Giemsa-stained thin smears the proportion of viable parasites with normal ring or trophozoite morphology at 66 h after drug removal *per* approx. 10,000 RBCs. Mean parasitaemia was determined and survival rates calculated as ratios of viable parasites in drug-exposed wells in comparison to unexposed controls. Results were considered interpretable when the parasitaemia in the control wells was higher than the starting parasitaemia.

2.7.6. Molecular biology methods

2.7.6.1. DNA extraction

Parasitic DNA was isolated from full blood aliquots and from filter paper spots through the QIAamp DNA mini kit (QIAGEN, Hilden, DE) according to the manufacturer's instructions. The DNA template was kept at −20°C until use.

2.7.6.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was established to amplify a particular gene or sequence of interest with specifically designed oligonucleotides. Sequence information were downloaded from databases www.plasmodb.org and www.genedb.org. Due to the high AT content in the genome of *Plasmodium falciparum*, oligonucleotides were designed in regions with possibly high GC content. One universal PCR protocol was used for all reaction with minor changes:

1 x Reaction buffer	Roche, Mannheim
200 µM dNTPs Mix	New England Biolabs GmbH, Frankfurt
2 mM $MgCl_2$	Roche, Mannheim
1µM Oligonucleotide each	Eurofins Genomics or Tib MOLBIOL, Berlin
2.5 U/50 µL Hot FIREPol	Solys Biodine, Tartu, Estonia

In few cases, reagents from the DNA Polymerase Kit: HotStarTaq DNA Polymerase (QIAGEN, Hilden, DE) were used. Genomic DNA was used as template. The final volume of the reaction was 25 µL or 50 µL depending on the subsequent experiments. Biometra Thermocyclers were used for the reaction using the cycling conditions listed in Table 2.2. A total of 35 or 40 cycles (Step 2-Step 4) for one reaction were the standard protocol. Extension time was adapted to template length (1 kb/1 min.).

PCR amplification was performed in order to align multiple sequences to detect point mutations (*e.g.*, in molecular markers of resistance such as *K13*, *pfdhfr* and *pfdhps*) as well as for *Plasmodium* species determination and identification of sub-microscopic infections. In the latter case, a nested PCR assay to amplify small subunit rRNA gene specific for each *Plasmodium spp.*, was performed as described elsewhere (WHO & CDC, 2010, Snounou *et al.,* 1993).

Denaturation of the DNA (Step 2)	0:45 min.	94°C
Annealing of the oligonucleotides (Step 3)	0:30 min.	58°C
Extension of the fragments by the *Taq polymerase* (Step 4)	1:00 min.	60°C
Final Extension to ensure complete amplification	10:00 min.	60°C
Pause		4°C

Table 2.2: Standard PCR cycling conditions. Conditions were modified/adjusted according to the fragment to amplify.

2.7.6.3. Agarose gel electrophoresis

Gel electrophoresis is a standard laboratory procedure that allows the distinct separation of DNA fragments by size (*e.g.*, length in base pairs) and their visualisation. To do so, the DNA fragments under analysis are loaded into an agarose gel matrix that is placed in an electrical field. The current generated moves the negatively charged DNA toward a positive electrode.

Briefly, agarose powder was added to 1 x TBE buffer in a microwavable flask and heated until complete dissolution, swirling the flask occasionally. In this work, different percentages of agarose (0.5-2%) were used depending on the size of the bands needed to be separated. A higher percentage of agarose creates a denser gel matrix indicated to separate smaller fragments while a lower percentage generates a gel with wider pores ideal for longer fragments.

After cooling the agarose solution down, ethidium bromide (EtBr) was added to a final concentration of approx. 0.2-0.5 µg/mL. EtBr binds to the DNA and allows its visualization under ultraviolet (UV) light. Agarose solution was then poured into a gel tray with a comb casted in. The comb forms small pockets (wells) in which the DNA is loaded once the gel has solidified.

After polymerization of the agarose, the gel tray was placed into an electrophoresis unit (gel box). The electrophoresis unit was filled with 0.5 x TBE buffer until the gel was completely covered and then the comb was carefully removed. A molecular weight ladder was loaded into the first lane of the gel and each sample was loaded into the remaining wells after addition of (6 x) loading buffer.

Afterwards, a current of 70-100V was applied and the gel was left to run until the dye line was approximately at the bottom of the gel. A picture was taken with SYNGENE Transluminator (Synoptics Ltd., USA).

2.7.6.4. PCR-Restriction Fragment Length Polymorphism (RFLP)

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) is a well-established method based on the use of restriction enzymes for the detection of single nucleotide polymorphisms (SNPs). Restriction enzymes have the capacity to recognize and cleave a DNA specific sequence (“restriction digest”).

The presence of the SNP is assessed through occurrence of a restriction site or its absence, which lead to different electrophoretic band patterns according to DNA fragments size (section 2.7.6.3.). This technique was used to assess polymorphisms in *pfdhfr* and *pfdhps*

in the Nigerian study (Study IV). Digestion was performed over night at 37°C using 0.3-1 U of enzyme.

Digestion of codon 108 was performed at 60-65°C as described somewhere else (Duraisingh *et al.*, 1998). Digestion conditions, restriction enzymes and expected fragment size are showed in table 2.3 and 2.4.

Digestion conditions	1x à 20µL	Final conc (Fc)
10x Cutsmart buffer	2 µL	1x
Enzym, NEB	According to enzyme	1 U
H_2O	8 µL	
Nested-PCR product	10 µL	

Table 2.3: Standard digestion conditions used for *pfdhfr* and *pfdhps* RFLP analysis. Enzyme final concentration and temperature of incubation where adjusted according to the enzyme in use.

Gene	Codon	Restriction enzyme	Expected fragment size
pfdhfr	51	*Mlu*CI	154 bp + 120 bp (Asn/wildtype)
			218 bp + 120 bp (Ile/mutation)
pfdhfr	59	*Xmn*I	189 bp + 137 bp (Cys/wildtype)
			163 bp + 137 bp (Arg/mutation)
pfdhfr	108 (a)	*Alu*I	320 bp + 196 bp (Ser/wildtype)
			522 bp (Asn/mutation **or** Thr/mutation)
pfdhfr	108 (b)	*Bsr*I	332 bp + 190 bp (Asn/mutation)
			522 bp (Ser/wildtype)
pfdhfr	108 (c)	*Bst*NI	320 bp + 196 bp (Thr/mutation)
			522 bp (Asn/mutation **or** Ser/wildtype)
pfdhfr	164	*Dra*I	245 bp + 171 bp + 107 bp (Ile/wildtype)
			245 bp + 143 bp + 107 bp (Leu/mutation)
pfdhps	436 (a)	*Mn*lI	278 bp + 121 bp + 39 bp (Ser/wildtype)
			317 bp + 121 bp (Ala/mutation)
pfdhps	436 (b)	*HindI*II	438 bp (Ser/wildtype **or** Ala/mutation)
			407 bp + 30 bp (Phe/mutation)
pfdhps	437	*Mwo*I	387 bp + 51 bp (Ala/wildtype)
			419 bp + 19 bp (Gly/mutation)
pfdhps	540	*Fok*I	405 bp + 33 bp (Lys/mutation)
			320 bp + 85 bp + 33 bp (Glu/mutation)
pfdhps	581	*Bst*UI	105 bp + 56 bp (Ala/wildtype)
			138 bp + 23 bp (Gly/mutation)

pfdhps	613 (a)	*Mwo*I	128 bp + 33 bp (Ala/wildtype)
			161 bp (Ser/mutation **or** Thr/mutation)
pfdhps	613 (b)	*Age*I	161 bp (Ala/wildtype **or** Ser/mutation)
			128 bp + 33 bp (Thr/mutation)

Table 2.4: Restriction enzymes and expected fragment size of *pfdhfr* and *pfdhps* RFLP analysis according to the protocol described by Duraisingh *et al.,* 1998.

2.7.6.5. DNA sequencing

Successfully amplified PCR products were bidirectionally sequenced by Source BioScience (Nottingham, UK) or by GATC from Eurofins Genomics (Berlin, DE). AB1 sequences were evaluated by the Blast search program on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure accuracy of PCR amplicons. SNPs of sample sequences were analysed in Bioedit v.7.0 by comparing with *P. falciparum* 3D7 strain sequence PF3D7_0523000 (*pfmdr1*) and PF3D7_1343700 (*K13*) retrieved from PlasmoDB (http://plasmodb.org/plasmo/).

Sequences from NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/) and NIH genetic sequence database GenBank (https://www.ncbi.nlm.nih.gov/genbank/) were used as reference for *pfdhfr* (XM_001351443.1) and *pfdhps* (Z30654.1) analysis, respectively. For *pvmdr1* multiple sequence alignment, *P. vivax* Sal-1 strain sequence (GenBank: AY618622.1) was used as reference. Mixed alleles were determined by the emergence of two chromatogram peaks at one nucleotide site through SnapGene v.3.1 (GSL Biotech LLC., Chicago, USA).

2.7.6.6. Data analysis

Patients were considered for analysis if they had microscopically visible and PCR confirmed parasitaemia and, for admitted patients, if malaria diagnosis was available within 24 hours. Data were established using Microsoft Excel 2017 and analyzed by SPSS v.22 (IBM Corp., Armonk, NY, USA) and Statview v.5.0 (SAS Institute Inc., Cary, NC, USA). Continuous parameters were compared between groups by Student's t-test, analysis of variance (ANOVA), Mann-Whitney U-test, or Kruskal-Wallis test as applicable. Proportions were compared between groups by Chi-square (χ^2) test or Fisher's exact test, and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Logistic regression was used to calculate adjusted odds ratios (aORs). Independent predictors of severe malaria were calculated by logistic regression analysis including factors showing association with severe malaria at a

level of p-value (p) <0.10 and with backward removal of factors not associated in multivariate analysis (p>0.05). A p-value <0.05 was considered statistically significant.

3. Results

3.1. Study I: Prevalence of *K13* polymorphisms in *P. falciparum* isolates from southern Rwanda, 2010 - 2015

In 2006, artemether-lumefantrine (AL) was introduced in Rwanda as artemisinin-based combination therapy (ACT) of choice for the first-line treatment of uncomplicated *P. falciparum* infections. Each ACT consists of a potent and rapid-acting artemisinin derivative (ART) and a longer-acting partner drug (section 1.5.2.). Leading ACTs such as AL have shown exceptional antimalarial efficacy (Dorsey *et al.*, 2007, Uwimana *et al.*, 2019), but early signs of resistance to ART, manifested as delayed parasite clearance after therapy, have been seen in parts of southeast Asia (SEA) and resistance has been already reported to most ACT's partner drugs in that region (Dondorp *et al.*, 2009, Rosenthal, 2013).

In 2014, the molecular marker of ART resistance, *Kelch13* (*K13*) was discovered and, to date, around 200 non-synonymous mutations in the *K13* gene have been reported worldwide (WHO, 2018b). Few candidate mutations associated with ART resistance in SEA have already been observed in Africa but their role and distribution among the continent remain unclear. No data on *K13* variants were available from Rwanda at the time this study was conducted. Furthermore, already in 2012, our laboratory reported a certain allele pattern in the *P. falciparum* multi-drug resistance marker *pfmdr1* indicative of reduced lumefantrine susceptibility in the *P. falciparum* parasite population in Huye district, southern province of Rwanda (Zeile *et al*., 2012). In this study, we aimed at further investigating the potential emergence and spread of drug-resistant parasites in the same study area.

To do so, blood specimens from 255 community children aged under 10 years and largely with asymptomatic malaria, were examined for markers of drug resistance. *P. falciparum* isolates were obtained from three groups of children in 2010, 2014 and 2015 in order to follow ART resistance-mediating *P. falciparum* polymorphisms over time.

Briefly, the first sample collection was performed as part of a cross-sectional survey on the prevalence of common childhood diseases. Samples were collected from children under five years of age in the Huye sub-district *i.e.,* from communities of the rural Huye sector, at Sovu Health Centre, and at the District Hospital of Kabutare (KDH) (section 2.7.1.1.). A total of 749 children were examined: 545 children from communities, 103 from Sovu Health Centre and 101 from KDH. Clinical and socio-economic characteristics of the enrolled children have been described in detail elsewhere (Gahutu *et al*., 2011). Overall, 16.7% (125) of the 749 children were found by PCR to harbour *P. falciparum* parasites of which 11.7% (87) had microscopically detectable parasitaemia.

Study sub-groups from 2014 and 2015 included community children aged 6-10 years old attending primary school who were examined as part of a study on anthelminthic treatment efficacy (Sifft *et al.*, 2016). Children infection status was defined by PCR for 878 children. Participants median age was 8 years (range: 6–10) and 54.4% (478) were female (Geus *et al.*, 2019). The number of evaluable children at each school ranged between 28 and 116 (median, 71).

Eventually a total of 255 *P. falciparum* isolates collected in the 3 different periods in Huye district were examined for polymorphisms in the ART resistance marker *K13*. Isolates were re-confirmed to be *P. falciparum* positive by semi-nested PCR assays performed at the Institute of Tropical Medicine (ITM), Charité-Universitätsmedizin Berlin, as described elsewhere (section 2.7.6.4.). The *K13*-propeller domain sequence was amplified by previously published PCR assays (section 2.7.6.2.). PCR products were bidirectional sequenced by Source BioScience company (Nottingham, UK) and multiple sequence alignment was performed to detect *K13* point mutations using the software SnapGene v.3.1 (GSL Biotech LLC., Chicago, USA).

Of 255 isolates, 222 (87%) *K13* amplicons were successfully sequenced. The median age of the 222 children from whom the isolates were obtained was 8 years old (range: 0.3–11), and 51.8% (115/222) were girls. The geometric mean parasite density was 4,217 parasites/µL of blood (95% CI, range: 3,175–5,601/µL, 199 samples); 10.4% (23/222) of samples were sub-microscopic, that is, positive by PCR only. Fever (axillary temperature ≥37.5°C) was present in 20.8% (42/221) of the children.

Intake of antimalarials, generally AL, was stated for 6.7% (5/75, taken in the preceding 2 weeks) of children in 2010, and for 39.5% (32/81, taken in the preceding month) in 2014 (no data for 2015).

Five of the 222 *P. falciparum* isolates (2.3%) revealed single nucleotide polymorphisms (SNPs) in the *K13* propeller domain (Table 3.1). All detected polymorphisms were non-synonymous. Notably, there was a non-significant trend toward increasing prevalence of polymorphisms, that is: none in 2010, 2.5% in 2014, and 4.5% in 2015 (χ_2 trend = 3.3, p = 0.07).

Among the three *K13* polymorphisms detected in isolates from 2015, two, *i.e.*, P574L and A675V, are candidate mutations associated with ART resistance (WHO, 2018). The remaining three polymorphisms identified were novel (D648H, V555A, A626S). The presence of *K13* polymorphisms was not associated with age, sex, parasite density, fever, or pre-treatment (data not shown) or to any other relevant clinical or socio-economic feature (Tacoli *et al.*, 2016).

Year	Number of sequenced samples	Non-synonymous mutations, n (%)	Amino acid & nucleotide changes
2010	75	0	- - -
2014	81	2 (2.5)	V555A (GTA→GCA) A626S (GCA→TCA)
2015	66	3 (4.5)	P574L (CCT→CTT) D648H (GAT→CAT) A675V (GCT→GTT)

Table 3.1: Prevalence of *K13* mutations in *P. falciparum* isolates from southern Rwanda, 2010-2015. Two isolates collected in 2015 harboured non-synonymous SNPs associated with ART resistance in SEA, *i.e.*, P574L and A675V (Tacoli *et al.*, 2016).

3.2. Study II: Polymorphisms in AS+SP and AL markers of resistance in *P. falciparum* isolates from southern India, 2015

Sulfadoxine-pyrimethamine (SP) *plus* artesunate (AS) is recommended as first-line treatment for uncomplicated falciparum malaria in India since 2010. Nevertheless, in the past years AS+SP treatment failure has widespread in northeast India, exceeding the threshold for changing drug policy. Based on these results, in 2013 artemether *plus* lumefantrine (AL) has been introduced as the new first-line treatment in Indian north-eastern regions (Mishra *et al.*, 2014).

SP treatment failure rates vary greatly across India, as do the frequencies of associated mutations in the parasite *dihydrofolate reductase* (*pfdhfr)* and *dihydropteroate synthase* (*pfdhps*) genes (Shah *et al.*, 2011, Mohapatra *et al.*, 2014). In this study, we assessed point mutations in molecular markers of resistance to SP (*pfdhps* and *pfdhfr*) and to artesunate (*K13*) in the port city of Mangaluru, coastal south-western Karnataka. In addition, we assessed *P. falciparum pfmdr1* alleles to appraise the potential of lumefantrine as an alternative partner drug for ACT (in combination with artemether) in case SP resistance would start developing in the study area.

Between June and December 2015, 909 patients attending the out-patient department (OPD) of Wenlock Hospital were enrolled in a study on the clinical characteristics of malaria in Mangaluru (section 2.7.1.2.).

Plasmodium species were ascertained by light microscopy on Giemsa-stained thin blood smears at the hospital laboratory and by semi-nested PCR assay as described elsewhere (section 2.7.6.2.). *P. vivax* malaria (69.6%) predominated over mixed *P. vivax-P. falciparum* infection (21.3%) and *P. falciparum* malaria (9.0%). The geometric mean parasite density of *Plasmodium* infections was 3,412 parasites/µL of blood.

Of 276 patients infected with *P. falciparum* parasites, 138 isolates (50%) were randomly selected for the assessment of molecular markers. Of those, 112 (81.1%) were successfully typed for all alleles under study, including 53 mixed *P. vivax-P. falciparum* infections.

A total of 20.5% (23) participants originally came from Mangaluru city while 79.5% (89) were migrants. Migrants came in 33.9% (38) from the local Karnataka state, 27.7% (31) from northern/north-eastern Indian states and 17.9% (20) from other regions of India. Migration to Mangaluru occurred at a median of six months (range: 1-240 months) before presentation at the hospital.

The median age of the 112 patients whose isolates were investigated was 30.5 years old (range: 10-65) and 92% (103) were male. The socio-economic status was low (Gai *et al.*, 2018), and 69.6% (78) of patients were construction workers or daily laborers. The geometric

mean parasite density was 9,572 parasites/µL of blood (95% CI, range: 7,516-12,190/µL). Each 4.5% (5) of patients were admitted to ward or had severe malaria, respectively. Intake of antimalarials in the preceding 6 weeks was reported by 0.9% (2) of patients.

To detect point mutations in *pfdhfr* and *pfdhps*, forward and reverse sequencing was performed by GATC company (Berlin, DE) (section 2.7.6.5.). Sequencing results are shown in Table 3.2.

Gene	Allele or Genotype	Prevalence (%, *n*/112)
pfdhfr	wildtype	33.9 (38)
	double mutation (C59R-S108N)	66.1 (74)
pfdhps	wildtype	29.5 (33)
	single mutation (A437G)	45.5 (51)
	double mutation (A437G-K540E)	25.0 (28)
pfdhfr/pfdhps	wildtype	26.8 (30)
	dhfr wildtype + *dhps* single	6.3 (7)
	dhfr wildtype + *dhps* double	0.9 (1)
	dhfr double + *dhps* wildtype	2.7 (3)
	dhfr double + *dhps* single	39.3 (44)
	dhfr double + *dhps* double	24.1 (27)
K13	wildtype	100 (112)
pfmdr1	wildtype	0 (0)
	single mutation (184F)	98.2 (110)
	double mutation (184F-1246Y)	1.8 (2)

Table 3.2: Prevalence of *P. falciparum* drug resistance alleles and genotypes in Mangaluru, southern India, 2015. Notably, 24% of quadruple *pfdhfr/ pfdhps* mutants were detected but no *K13* mutations. *Pfmdr1* haplotype N86-184F-D1246 was highly prevalent (Wedam *et al.*, 2018).

Only one third of *pfdhfr* alleles were wildtype, and this figure was even lower for *pfdhps*. Two thirds of isolates exhibited the double mutation C59R-S108N in the *pfdhfr* gene while the single *pfdhps* mutation A437G (46%) dominated over the double variant A437G-K540E. Taken together, 44% of isolates showed *pfdhfr* C59R-S108N *plus* the *pfdhps* A437G mutation while *pfdhfr*/*pfdhps* quadruple mutants (C59R-S108N/A437G-K540E) were present one in four isolates.

Isolates from native Mangaloreans and from migrants did not differ significantly ($p = 0.78$) in terms of prevalence of *pfdhfr/pfdhps* triple or quadruple variants, which, together, were detected in 60.9% (14/23) and in 64% (57/89) of the isolates, respectively.

Of note, as compared to patients from Mangaluru, the proportion of *pfdhfr/pfdhps* triple or quadruple mutants did not significantly differ among migrants from north/north-eastern Indian states (67.7%, 21/31; p = 0.6), but tended to prevail in migrants from the local state of Karnataka (78.9%, 30/38; p = 0.13), and it was significantly reduced in migrants from elsewhere in India (30.0%, 6/20; p = 0.04). The time since migration was not associated with carrying SP-resistant parasites (p = 0.74).

As for the molecular marker of ART resistance *K13*, no polymorphisms were detected among the 112 Indian isolates screened.

With respect to *pfmdr1* alleles reflecting tolerance to the alternative ACT partner drug lumefantrine, we identified only the wildtype allele at the central codon 86 of *pfmdr1* (N86), and almost exclusively in the haplotype combination 86N-184F-1246D (called NFD).

3.3. Study III: Prevalence of *pvmdr1* polymorphisms in *P. vivax* isolates from Mangaluru, southern India, 2015

Current treatment of *P. vivax* relies upon two main antimalarial drugs: chloroquine (CQ) which acts on the parasites' blood stage, and primaquine, the most effective drug to eliminate latent liver stage infections and prevent relapse (WHO, 2018).

CQ-resistant vivax malaria has already reached an alarming prevalence in SEA and it is spreading in Northeast India. Unlike in *P. falciparum*, the molecular mechanism of CQ resistance in *P. vivax* parasites remains elusive (Price *et al.*, 2009). Nevertheless, such resistance is thought to be mediated by single nucleotide polymorphisms (SNPs) in the multidrug resistance gene *pvmdr1*, orthologous of *P. falciparum's pfmdr1* (Brega *et al.*, 2005).

In the current study, we aimed at assessing the presence of mutations associated with CQ resistance in the *P. vivax* molecular marker *pvmdr1*, in the costal city of Mangaluru, Karnataka state, southern India.

Like for Study II, blood samples were collected from 909 malaria patients attending Wenlock Hospital in June-December 2015. Six-hundred thirty-three patients over 909 had *P. vivax* mono-infections and were treated with CQ for 3 days *plus* primaquine for 14 days. Patients investigated were mostly young (median age: 25 years old), males (93%) and presented a geometric mean parasite density of 2,999 parasites/µL of blood (95%CI, range: 2,660-3,382/µL). CQ intake within the four weeks preceding presentation was stated for <1% of patients. Further clinical and socio-economic features of the enrolled patients are described elsewhere (Gai *et al.*, 2018). Study participants were asked to return to the hospital on Day-2 (48 h) or Day-3 (72 h) of CQ treatment to evaluate infection clearance by parasite count on Giemsa-stained thick blood films by microscopy.

Among the 633 *P. vivax* patients recruited, 18.3% (116) returned for the recommended control of which 12.7% (81) on Day-2 and 5.2% (33) on Day-3. Two further patients came back at days 4 and 5. In order to perform a comparison between mutations in the selected molecular marker and clearance on Day-3 of CQ treatment, *pvmdr1* polymorphisms were assessed only on blood specimens collected from patients returning late on.

For *pvmdr1* typing, the *pvmdr1* sequence was amplified as published elsewhere and PCR products were bidirectional sequenced by GATC company (Berlin, DE) (section 2.7.6.2.). *Pvmdr1* sequencing was successful for 108 isolates (93.1%, 108/116). Four synonymous point mutations, *i.e.*, T529T, A970A, S1358S, R1422R and eight non-synonymous polymorphisms, that is, S513R, T958M, Y976F, F1076L, Y1028C, L1393N, L1425R and T1269S were identified in the present study. All 108 *P. vivax* isolates presented the synonymous point mutation at codon 529 and the non-synonymous (ns) SNP T958M. Of these, 87.0% (94/108) additionally had the substitution F1076L. The synonymous

polymorphisms at position 970, 1358 and 1422 were detected in 1.9% (2/108), 8.4% (9/108) and in 0.9% (1/108) of the *P. vivax* isolates investigated, respectively. After the mutations T958M and F1076L, the most prevailing nsSNPs were 1393N, which was present in 26 patients (24%), and S513R, detected in 10 isolates (9.6%). The other non-synonymous polymorphisms were sporadically detected in few patients only, *i.e.,* Y976F (0.9%, 1/108), Y1028C (2.8%, 3/108), L1425R (0.9%, 1/108), and T1269S (3.7%, 4/108).

Of note, *P. vivax* isolates harbouring substitution F1076L did not carry mutations 1269S and 1393N in an almost mutually exclusive manner ($p < 0.001$). *Vice versa*, the nsSNP S513R did only occur among parasites carrying the mutation F1076L.

Ten *pvmdr1* haplotypes were recognized in total (Table 3.3) including the quadruple mutation S513R-T958M-Y976F-F1076L that was observed in 1 isolate (0.9%).

***Pvmdr1* haplotype**	**No.**	**%**	**Proportion of patients parasitemic on Day-2 or Day-3 of CQ treatment**
S513R-T958M-Y976F-F1076L	1	0.9	1/1 (100%)
S513R-T958M-Y1028C-F1076L	3	2.8	0/3 (0%)
S513R-T958M-F1076L-L1393N	2	1.9	0/2 (0%)
S513R-T958M-F1076L-L1425R	1	0.9	0/1 (0%)
T958M-F1076L-T1269S-L1393N	1	0.9	1/1 (100%)
T958M-F1076L-T1269S	3	2.8	1/3 (33.3%)
S513R-T958M-F1076L	3	2.8	0/3 (0%)
T958M-F1076L-L1393N	9	8.3	0/9 (0%)
T958M-L1393N	14	13	1/14 (7.2%)
T958M-F1076L	71	65.7	6/71 (8.5%)

Table 3.3: Prevalence of *pvmdr1* haplotypes and proportion of parasitemic patients upon follow-up in *P. vivax* cases from Mangaluru, 2015. Mutation Y976F was identified only in one isolate collected from one patient still positive on Day-2 of ACT treatment (no data available on Day-3) (Tacoli *et al.*, 2019).

For what concerns parasite clearance, on Day-2 of CQ treatment, 87.7% (71/81) of patients presenting for the check-up had successfully cleared parasitaemia, and this figure was 93.9% (31/33) on Day-3. Two further patients were free of malaria parasites when presenting on days 4 and 5 of treatment. None of the individual *pvmdr1* polymorphisms (data not shown) or haplotypes were associated with Day-2 or Day-3 positivity.

3.4. Study IV: SP resistance in pregnant women in Calabar, Nigeria, 2013-2014

Malaria in pregnancy is a major public health problem, with substantial risks for the mother, the fetus and the newborn. In high transmission areas, its prevention in pregnancy is based on intermittent preventive treatment (IPTp). IPTp consists in the administration of a single curative dose of an efficacious antimalarial drug at best on a monthly base, regardless of whether the woman is infected or not. The drug is administered under supervision during antenatal care (ANC) visits. Sulphadoxine-pyrimethamine (SP) is the drug currently recommended by the WHO.

IPTp with SP (IPTp-SP) is very effective in reducing the adverse outcomes of falciparum malaria in pregnancy but is threatened by the emergence of drug-resistant parasites (McGready *et al.*, 2011, Cottrell *et al.*, 2015).

Previous studies have already reported resistance to SP in Calabar, southeast Nigeria, to be over 80% (Ezedinachi *et al.*, 1992). The present study aimed at confirming the presence of SP resistance in this area, by determining the prevalence of molecular markers of SP resistance (*i.e.*, *pfdhfr* and *pfdhps*) in *P. falciparum* isolates obtained from asymptomatic pregnant women attending the General Hospital of Calabar.

Isolates of *P. falciparum* were collected as part of a clinical trial (registration No: PACTR201308000543272) performed between October 2013 and November 2014 on the effectiveness of intermittent screening and treatment for malaria prevention in pregnancy.

Briefly, the trial investigated whether screening pregnant women for malaria with a rapid diagnostic test during routine ANC visits and treating those found positive with AL was less effective than following IPTp-SP (trial details available at the Pan African Trials Registry: https://pactr.samrc.ac.za/Search.aspx).

Asymptomatic pregnant women attending their first ANC visit who had not received any previous dose of IPTp-SP were asked to participate to the study. A total of 460 pregnant women in their 16–24 weeks (4^{th}-6^{th} month) of gestation gave their consent.

Asymptomatic infection with *P. falciparum* parasites were detected by microscopy on Giemsa-stained thick blood smears in 7% (32/459) of the screened pregnant women at the time of their first ANC visit. Only these samples were considered to investigate mutations in *pfdhfr* and *pfdhps* molecular markers. *Pfdhfr* and *pfdhps* amplification by PCR and subsequent digestion by restriction enzymes (section 2.7.6.5.) was successfully obtained in twenty-eight samples (87.5%) out of 32.

The mean age of the 28 asymptomatic pregnant women was 27.3 (±4.3) years old and about 46% (13/28) of them were primigravidae (*i.e.*, at their first pregnancy). Ownership of bed nets was 46.4% (13/28) but less than half of the women (6/13) slept under a bed net the

night before enrolment in the study and blood sampling (Table 3.4). Women who reportedly slept under a bed net the night before did not have significantly lower parasite densities compared to those who did not (p= 0.748). Overall, the mean of haemoglobin level was 10.1 ±1.4 g/dL. Parasite density was generally low.

Categories	Variables	n (%)
Educational attainment	Primary	3 (10.7)
	Secondary	9 (32.1)
	Tertiary	16 (57.2)
Gravidity	Primigravidae	13 (46.4)
	Secundigravidae	9 (32.2)
	Multigravidae	6 (21.4)
Ownership of bed net	Yes	13 (46.4)
	No	15 (53.6)
Slept under bed net (previous night)	Yes	6/13 (46.2)
	No	7/13 (53.8)
Haemoglobin (g/dL) level	11 and above	6 (21.4)
	8–10	19 (67.9)
	< 8	3 (10.7)
Parasite density	parasite/µL, median [interquartile range])	768.5 [256–2799]

Table 3.4: Characteristics of pregnant women positive for asymptomatic malaria infection at enrolment. Half of the screened subjects were at their first pregnancy. Interestingly, the use of bed nets did not significantly affected parasitaemia (Esu *et al.*, 2018b).

All *P. falciparum* isolates harboured the *pfdhfr* core mutation S108N. Additional *pfdhfr* substitutions at codon 51 (N51I) and 59 (C59R) were detected in 92.9% (26/28). The high resistance mutation *pfdhfr* I164L was not found.

Pfdhps mutations were detected in 82.1% (23/28), 96.4% (27/28), 71.4% (20/28) and 71.4% (20/28) of *P. falciparum* isolates for codons 436 (S436A), 437 (A437G), 581 (A581G) and 613 (A613S), respectively. None of the samples carried the *pfdhps* mutation K540E.

Table 3.5 shows the prevalence of *pfdhfr/pfdhps* mutant alleles and haplotypes. A single mutation in the *pfdhfr* gene at codon 108 (S108N) was detected in only one isolate (3.6%). A double mutation, made up of single mutations in the *pfdhfr* and *pfdhps* genes (S108N and 437G), was found in one sample (3.6%). Also, the frequency of quadruple (N51I-C59R-S108N/437G) and quintuple (N51I-C59R-S108N/S436A-A437G) *pfdhfr/pfdhps* mutants were of 10.7% (3/28) for both.

The prevalence of triple *pfdhfr* mutation (p = 0.436) and of quadruple (p = 0.144), quintuple (p = 0.11) and septuple (p = 0.871) *pfdhfr/pfdhps* mutants was not significantly lower in primigravidae compared to secundi- and multi-gravidae women.

Similarly, the prevalence of *pfdhfr* and *pfdhps* mutant alleles and haplotypes did not differ significantly between highand low-density malaria infections (data not shown).

Gene	Allele or Genotype	Prevalence (%, *n*/28)
pfdhfr	Single mut (S108N)	7.2 (2)
	Triple mut (N51I-C59R-S108N)	92.8 (26)
pfdhps	Wildtype	3.6 (1)
	Single mut (437G)	14.3 (4)
	Double mut (S436A-A437G)	10.7 (3)
	Quadruple mut (S436A-A437G-A581G-A613S)	71.4 (20)
pfdhfr/pfdhps	*dhfr* single mut + *dhps* wildtype	3.6 (1)
	dhfr single mut + *dhps* single mut	3.6 (1)
	dhfr triple mut + *dhps* single mut	10.7 (3)
	dhfr triple mut + *dhps* double mut	10.7 (3)
	dhfr triple mut + *dhps* quadruple mut	71.4 (20)

Table 3.5: Prevalence of *pfdhfr* and *pfdhps* mutant alleles and haplotypes. Notably, 71.4% of isolates was carrying a *pfdhfr/pfdhps* septuple mutant. Mut = mutation (Esu *et al.*, 2018).

3.5. Study V: High *ex vivo* RSA survival rates in *P. falciparum* isolates from southern Rwanda, 2018

After the WHO's recommendation (WHO, 2001) to use ACTs for the treatment of uncomplicated falciparum malaria, the burden of disease worldwide has declined substantially. Nevertheless, as with previously used antimalarial drugs (Wongsrichanalai *et al.*, 2002), parasite resistance to artemisinin and its derivatives (ART) has emerged in Southeast Asia.

The identification of *K13* as markers for ART resistance has allowed for a more refined definition of partial ART resistance that includes information on the parasite genotype. However, not all of the non-synonymous propeller-region *K13* mutants reported indicate the emergence of ART resistance. This is particularly true in Africa where such mutants can represent 'passer-by' genotypes in the absence of evidence for the selection of the mutant *K13* genotype (WHO, 2018b). The validation of a *K13* mutation as a marker for ART resistance requires its correlation to slow clearance infections *in vivo* expressed as parasite clearance half-life (PCHL) after exposure to ART greater than 5 hours (section 1.5.2.)

An alternative reliable method to assess slow clearance infections *ex vivo* is represented by the ring-stage survival assay (RSA). High *ex vivo* RSA survival rates expressed as ≥10% ratio of viable ART-exposed parasites to unexposed controls (section 2.7.5.6.) are considered to reflect slow clearance infections *in vivo* in a highly specific and sensitive manner (89% specificity and 91% sensitivity) and are therefore a valuable tool for clearance assessment when multiple parasite counts to determine PCHL are not feasible (Ikeda *et al.*, 2018).

In the present observational, non-interventional study we aimed at assessing the prevalence of slowly clearing infections in patients with *P. falciparum* malaria from the Huye district of southern Rwanda. For this purpose, we performed *ex vivo* RSA on freshly collected isolates and investigated the correlation between the high parasite survival rate observed in the assay and the full clearance of the parasite from patient's peripheral blood after 3 days regimens of arthemeter-lumefantrine (AL) administration. Furthermore, we assessed the presence and distribution of mutations in the molecular marker of ART resistance *K13* to gain a comprehensive overview of the extension of putative ART resistance in the study area.

The research study was conducted between March and May 2018 at 4 locations within the Huye district *i.e.*, at Sovu Health Centre, Rukira Health Centre (branch of Sovu), at Kabutare District Hospital (KDH) and at the University Teaching Hospital of Butare (*Centre Hospitalier Universitaire de Butare* or CHUB). A total of 210 patients with uncomplicated *falciparum* malaria and 4 with severe malaria were recruited in the study.The average age of the enrolled patients was 23 years (range: 10-32) and a similar proportion of males and females were recruited (55% males, 45% females).

The *ex vivo* RSA was performed on uncultured parasite isolates within 24 hours from collection. Briefly, freshly collected parasite isolates were exposed to pharmacologically relevant concentrations of DHA for 6 h and survival rates were assessed by microscopy after recovery by counting the proportion of viable parasitaemia in comparison to non-exposed isolates (DMSO controls) (section 2.7.5.6.). High *ex vivo* RSA survival rates were expressed as a ratio of viable DHA-exposed parasites equal or greater than 10% as compared to DMSO controls in accordance with WWARN guidelines (Witkowski *et al.*, 2013).

Among the 214 patients enrolled in the study, 35 isolates (35/214, 16.4%) were considered suitable for the *ex vivo* RSA. Due to the lack of a donor of uninfected erythrocytes, only isolates with an initial parasitaemia of <1% were processed.

Ex vivo RSA survival rates were assessed for a total of 35 isolates *i.e.*, twelve isolates obtained in March, 11 in April and further 12 isolates in May. Of the processed samples, 9 *P. falciparum* isolates presented survival rates greater than 10% (9/35, 25.7%) with a mean survival rate of 25% (min. 11% - max. 47%) (Figure 3.1).

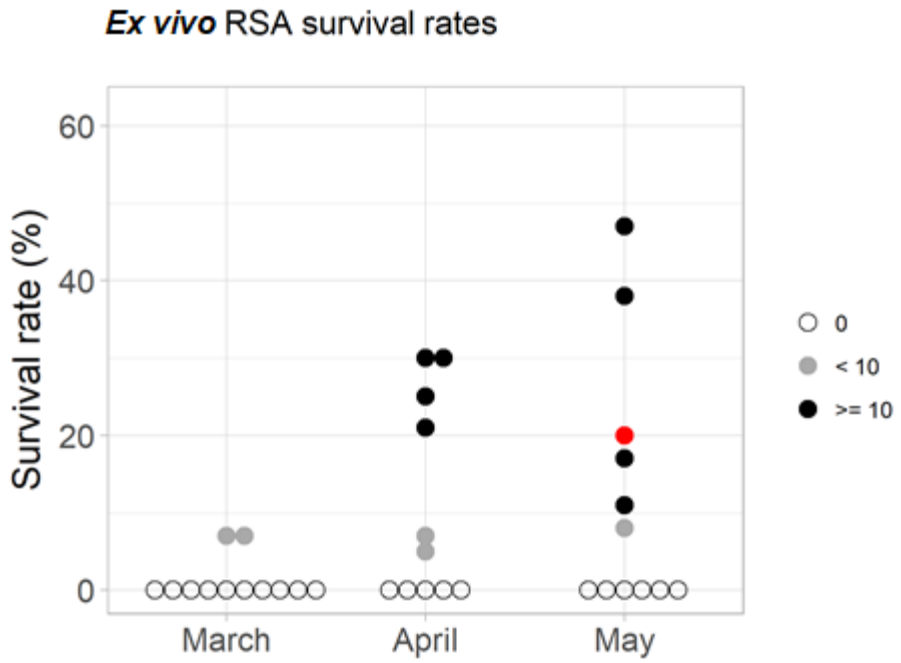

Figure 3.1: *Ex vivo* RSA survival rates observed in samples collected in March-May 2018 in the Huye district. Approx. twelve isolates were processed per month. Samples are represented with black, light-grey or white dots when *ex vivo* RSA survival rates were greater or equal to 10%, between 0% and 10%, or equal to 0, respectively. One patient recruited in May (red dot) was exhibiting a detectable parasitaemia on Day-3 of ACT and presented high RSA survival rate (20%) (Data not published).

Importantly, two isolates exhibit the highest *ex vivo* RSA survival rates ever reported in Africa with 38% and 47%, respectively as compared to 34.3% observed in Uganda (Ikeda *et al.*, 2018). Parasite isolates collected in April and May where overall more resistant (higher

number of samples with survival rates >10%) to DHA exposure than those processed in March. A total of 137 *falciparum* malaria patients (64%) returned on Day-3 for parasite clearance assessment, including 80% (24/35) of the patients whose isolates were process by *ex vivo* RSA. Among the isolates collected from the latter, 33.3% (8/24) showed a survival rate greater than 10%.

Notably, one isolate with an *ex vivo* RSA survival rate of 20% (red dot in Figure 3.1) was collected in May from a thirteen year old individual presenting with high fever (39.8°C) and mild malnutrition (MUAC = 19.5). Additional malaria-related symptoms were chills, muscular pain, headache, weakness, nausea, loss of appetite and abdominal pain. The patient, whose initial uptake of the prescribed ACT was observed, had not fully cleared *P. falciparum* infection on Day-3 of treatment presenting a residual parasitaemia of 240 parasites/µL of blood (0.005%).

The other patients returning on Day-3 of ACT did not present remarkable malaria-related symptoms with the exception of three patients that were diagnosed with mixed morbidity at the time of enrolment and still presented signs of headache and loss of appetite among others (data not shown).

Furthermore, venous blood collected from returning patients was shipped to the Institute of Tropical Medicine (ITM), Charité-Universitätsmedizin Berlin, for *K13*-propeller domain amplification and bidirectional sequencing (Eurofins Genomics, Berlin, Germany).

Among the 136 isolates analyzed, two non-synonymous polymorphisms were identified, that is, P574L and C469F, which are candidate mutations associated with ART resistance in Southeast Asia. Interestingly, the isolate harbouring mutation P574L was detected in May in a four years old child who successfully recovered after 3 days of treatment and did not show high e*x vivo* RSA survival rates (survival rate = 2%). The isolate carrying mutation C469F was not processed by RSA, but the patient infected by this mutant parasite recovered as well after the 3 days regimens of ACT.

4. Discussion

4.1. Detection of candidate mutations in *K13*-propeller domain associated with ART resistance in Huye, southern Rwanda, in 2015

To date, *in vivo* therapeutic efficacy studies (TES) and *in vitro* and *ex vivo* drug testing are considered the gold standard for determining antimalarial drug efficacy. However, WHO recommends these studies to be complimented with molecular surveillance using genetic markers associated with resistance (WHO, 2009b). Mutations in the *P. falciparum K13* gene, especially in the propeller-domain sequence, have been associated with delayed parasite clearance *in vivo* and higher survival rates *in vitro* upon ART exposure and are therefore a valuable tool for rapidly detecting and tracking evolutionary patterns of resistance in the parasite population (Ariey *et al.*, 2014).

A list of the currently known *K13* mutations associated with ART resistance, which are either still candidate markers or have already been validated is provided in table 4.1.

In this study, we tracked the presence of putative ART-resistant parasites in the southern province of Rwanda by genotyping the *K13*-propeller region in 222 *P. falciparum* isolates collected between 2010 and 2015 from school and community children. We showed that *K13* mutations are present in southern highland Rwanda at a low frequency and that among these mutations there are two candidate polymorphisms previously observed in SEA and associated with ART resistance, namely A675V and P574L.

VALIDATED		CANDIDATES/ASSOCIATED	
F446I	**P553L**	P441L	G538V
N458Y	**R561H**	G449A	V568G
M476I	**C580Y**	C469F	P574L
Y493H		A481V	F673I
R539T		P527H	A675V
I543T		N537I	

Table 4.1: Candidate and validated *K13* mutations associated with artemisinin resistance in SEA. (WHO, 2018b).

Variants A675V and P574L are common in Southeast Asia (SEA) and have been widely associated with delayed parasite clearance (Ariey *et al.,* 2014, Miotto *et al*., 2015, Amato *et al*., 2016, WHO, 2018). In a recent study performed by the *K13* Genotype-Phenotype Study Group in Asia, patients infected with *P. falciparum* harbouring *K13* variants P574L and A675V

had 1.8 to 2.4-fold increase in the geometric mean of parasite clearance half-life (PCHL), respectively, compared to wildtype parasites collected at the same sites (WWARN, 2019). For P574L and A675V, the median PCHL values reported were 5.9 h and 6.7 h, respectively (Figure 4.1). These data support the hypothesis that both mutation P574L and A675V are strongly associated to *P. falciparum* ART-resistant phenotype.

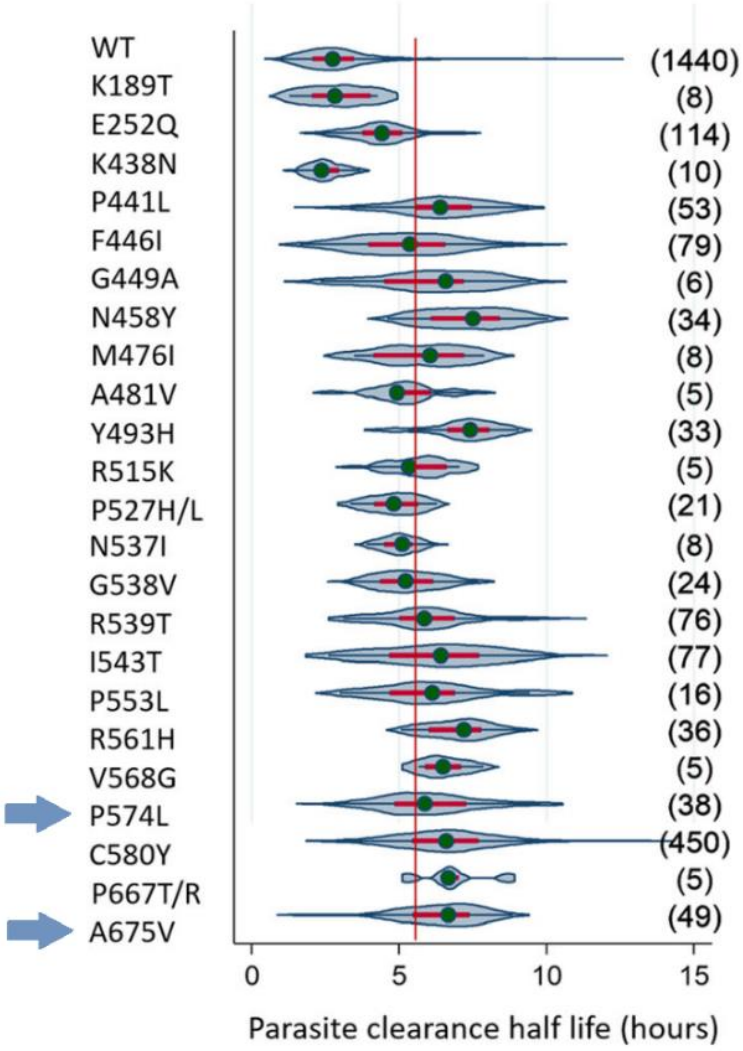

Figure 4.1: Distribution of parasite clearance half-life (PCHL) by *K13* variants in isolates from Asia. P574L and A675V are highlighted by blue arrows. The numbers in parentheses on the right refer to the amount of patients screened to assess PCHL for each mutation. Figure adapted from WWARN, 2019.

Nevertheless, the actual role played by *K13* polymorphisms in sub-Saharan Africa is a long way from being understood. So far, few *K13* polymorphisms associated with delayed parasite clearance in SEA have been detected in Africa. In one survey across 14 African sites, 1,184 *P. falciparum* isolates were collected between 2002 and 2011. Among these, 23 different *K13* mutations (15 coding ones) were observed, of which 18 restricted to single geographical sites.

Two candidate mutations (the latter now validated) were identified, namely G449D and P553L in Mali and in Kenya, respectively (Taylor *et al.*, 2015). Another large-scale study on 1,212 African *P. falciparum* isolates collected in 2013–2014 found 22 *K13* polymorphisms (7 non-synonymous) at allele frequencies of 1–3% (Kamau *et al.*, 2015). In 2015, the assessment of *K13* variants by the MalariaGEN consortium revealed 64 *K13* polymorphisms in 1,648 African samples. Among the 26 non-synonymous mutations detected in the study, 14 were previously reported in isolates from SEA, and seven of those are considered to confer ART resistance (Amato *et al.*, 2016).

Studies on *P. falciparum* neutral microsatellite loci have revealed that ART-resistant *K13* variants have distinct geographic origins in SEA and are therefore a consequence of local evolution. For instance, R539T mutants have originated at the China-Myanmar border and at the Thai-Cambodian/Thai-Myanmar borders while the F446I mutation is the most common mutation in isolates from China, China-Myanmar border and northern Myanmar but remains rare in the rest of SEA (Ye *et al.*, 2016, Tun *et al.*, 2016). In this same way, the majority of *K13* polymorphisms identified in Africa appeared to be of local origin, and were substantially more heterogeneous as compared with isolates from SEA.

In line with this notion, the analysis of African isolates suggests the possibility of a neutral evolution of the rare *K13* polymorphisms, and the presence of a large reservoir of "natural" *K13*-propeller variants in sub-Saharan Africa instead of a drug pressure selection of these mutations (Amato *et al.*, 2016). This hypothesis was corroborated by the lack of association between African *K13* mutants and delayed parasite clearance *in vivo* observed in a data-meta analysis performed by the WWARN *K13* Genotype-Phenotype Study Group, where 204 *P. falciparum* isolates from five sites in Africa were evaluated for their association between *K13* genotype and parasite clearance phenotype. A total of 7 *K13*-propeller variants were detected, 3 in the Democratic Republic of Congo and 4 in Madagascar, but all patients infected with *K13* mutants had a PCHL <3 h (WWARN, 2019).

In contrast with this figure, other monitoring studies seem to support the hypothesis that *K13* variants may play a role in the emergence of ART resistance in Africa as well. At the time the study was performed, the *K13* variant A675V had been detected only once among more than 4,000 African *P. falciparum* isolates (Amato *et al*., 2016, Kamau *et al*., 2015, Taylor *et al.*, 2015) while *K13* candidate mutation P574L was reported here for the first time from Africa. In 2018, the A675V mutation, which we observed in Rwanda, has been detected for the first time in association with high (≥10%) *ex vivo* RSA survival rates in northern Uganda, becoming the first *K13* variant potentially associated with ART resistance in both SEA and Africa (Ikeda *et al.,* 2018).

Furthermore, the first case of indigenous ART-resistant *P. falciparum* from Africa has been recently reported by Lu and co-workers. A fourty-three years old man from China had a recrudescence of malaria approx. one year after returning from Equatorial Guinea, where 14

months before he had a *P. falciparum* infection treated with parenteral artesunate monotherapy (Lu *et al*., 2017). The patient received a full course of DHA+PPQ for 3 days under observation but parasites were still detected on Day-3 of treatment up to Day-7. *K13* genotyping of the isolate pointed out the presence of a previously unreported non-synonymous single-nucleotide polymorphism (nsSNP) that resulted in a switch from a methionine to an isoleucine at position 579 (M579I). Based on these data, each *K13* mutation detected in Africa may be relevant for the emergence of ART resistance.

To date, the mechanisms underlying the selection of *K13* alleles in a parasite population remains poorly understood. For instance, the reason why the ART-resistant *K13* mutation C580Y has remarkably spread in multiple countries of SEA is still unclear. *P. falciparum* isolates carrying this mutation do not present significantly higher survival rates upon exposure to ART *in vivo* (measured as PCHL) as compared to other ART-resistant *K13* substitutions (Anderson *et al.*, 2016). Also, patients infected with parasites bearing C580Y show risks of treatment failure comparable to those of patients infected with parasites bearing other *K13* ART-resistant variants. Moreover, parasites carrying the *K13* allelle C580Y do not seem to produce more gametocytes than those bearing other ART-resistant *K13* alleles (Phyo *et al.*, 2016). In a recent *in vitro* study, it was shown that the mutation C580Y not only carries higher fitness costs for the parasite in the absence of drug pressure but also does not outcompete other mutants associated with ART resistance in direct *in vitro* competition (Nair *et al.*, 2018).

The degree of ART resistance observed in *P. falciparum* parasites harbouring validated *K13* mutations seems to be strain-specific, *i.e.*, to relate to the genetic background of the parasite (Ghorbal *et al.*, 2014, Straimer *et al.*, 2015). Thus, variants elsewhere in the genome may be needed to compensate for the deleterious effects of *K13* mutations on the parasite's fitness. In many pathogens, mutations conferring resistance to drugs also confer deleterious fitness effects that are usually suppressed or mitigated by co-segregating compensatory mutations, a phenomenon well documented in bacteria (Bjorkman *et al.*, 1998), yeast (Cowen *et al.*, 2001), and *P. falciparum* (Jiang *et al.*, 2008, Nair *et al.*, 2008, Petersen *et al.*, 2015). Few studies in this direction have been conducted, pointing out additional non-*K13* genetic factors in the local parasite population the expression of which varies in diverse genetic strains and may predispose to the emergence of resistance (Cheeseman *et al.*, 2015). A genome-wide association study performed by Miotto and co-workers has pointed out few polymorphisms that may correlate with ART resistance such as the substitution D193Y in the ferredoxin protein, mutation T484I in the *P. falciparum* multidrug resistance protein 2 (PfMDR2) and mutations V127M, V1157L and C1484F in the apicoplast ribosomal protein S10 (Arps10), the protein phosphatase (Pph) and the phosphoinositide-binding protein (Pibp), respectively (Miotto *et al.*, 2015). Additionally, it has been suggested that the link with resistance may differ geographically (Kamau *et al.,* 2015).

The fitness costs of the *K13* variants may prevent their *de novo* establishment or/and spread in African parasite populations. This is particularly true in regions with high transmission of malaria where multiple infections are common, *i.e.,* emerging *K13* mutations associated with ART resistance must constantly compete with co-infecting wildtype parasites with higher fitness.

Furthermore, the lower levels of disease endemicity and acquired immunity in SEA strongly increase the likelihood that infected individuals will become symptomatic and thus will be treated with drugs. In Africa most infections are asymptomatic, hence the proportion of the parasite population exposed to ART selection is lower (Cerqueira *et al.*, 2017, Nair *et al.*, 2018).

The prevalence of many diverse individual but overall rare non-synonymous polymorphisms in Africa likely reflects the only recent and not universal access to ACTs and thus limited drug pressure and selection as compared with SEA, where ACTs have been in use for much longer, offering a wider window of time for background mutations to occur and rise in frequency (White *et al.*, 2015).

Moreover, Southeast Asian parasites experience a lower rate of sexual outcrossing than parasites in most African populations. This is because *P. falciparum* is an obligately sexual but facultatively outcrossing eukaryotic parasite. *P. falciparum* meiosis occurs following the union of parasite gametes in the mosquito midgut (section 1.2.1). In low transmission regions like SEA, the majority of mosquitoes feed on humans infected by a single parasite genotype that will result in self-fertilization of male and female gametes (Nkhoma *et al.*, 2013), leading to infrequent recombination as compared to high transmission settings like sub-Saharan Africa, where human infections may contain multiple parasite genotypes (Nwakanma *et al.*, 2008). The high levels of sexual outcrossing between unrelated parasite genotypes occurring in Africa disrupt associations with potential compensatory alleles elsewhere in the genome (Anderson *et al.*, 2000).

Nevertheless, the potential emergence and spread in Africa of optimized *P. falciparum* genotypes from SEA containing a *K13* mutation associated with ART resistance coupled with compensatory changes is a real risk, thus, the routine monitoring and surveillance of *K13* variants together with informative clinical studies should be performed to facilitate early detection of putative resistance foci.

Among the three novel *K13* polymorphisms identified in our study, V555A was one of five polymorphisms detected among isolates from different areas of Rwanda in the KARMA (acronym for *K13* Artemisinin Resistance Multicentre Rapid Assessment) Consortium project. In those isolates collected in 2012–2013, no candidate mutation associated with ART resistance was observed (Ménard *et al.*, 2016).

The proportion of the *K13* polymorphisms we detected, tended to increase over time. Although derived from a rather small single-centre study, this observation may reflect the

increased availability of ACTs in Rwanda during recent years. Moreover, the incidence of malaria in this country has increased since 2011 possibly increasing the likelihood of the random occurrence of *K13* variants (PMI, 2016).

Already in 2010, we observed a *pfmdr1* allele constellation in the study area (40% *pfmdr1* N86-F184-D1246, section 1.6.3.), which is indicative of intense AL drug pressure and reappearing parasitaemia following treatment (Zeile *et al.*, 2012). Against such a background of a parasite population with affected susceptibility to the non-artemisinin partner drug, the potential of spreading *K13* candidate mutations is worrisome.

4.2. Molecular evidence for *P. falciparum* resistance to sulfadoxine-pyrimethamine but absence of *K13* mutations in Mangaluru, southern India, in 2015

In the current study, we showed that in coastal south-western India, the majority of *P. falciparum* isolates have mutations conferring a certain degree of SP resistance, while *K13* variants associated with ART resistance are absent. In particular, we showed that 39.3% of isolates carried the triple mutant *pfdhfr/pfdhps* C59R-S108N/A437G and 24.1% the quadruple mutant C59R-S108N/A437G-K540E. Although no quintuple or sextuple mutants were observed, in the light of the intensification of SP resistance seen elsewhere in India (Shah *et al.*, 2011), the useful therapeutic lifetime of the current combination AS+SP appears limited.

Isolates with increased resistance to sulfadoxine emerge only after a substantial fraction of the population had been selected for pyrimethamine resistance (Mberu *et al.*, 2000). In fact, the prevalence of *pfdhps* A437G and K540E only in parasites carrying the double *pfdhfr* mutation provides evidence for such positive directional selection of a specific *pfdhfr/pfdhps* haplotype towards resistance (Wang *et al.*, 1995). Such a process, where natural selection acts to increase the frequency of a beneficial allele to fixation in the population, is usually referred to as selective sweep. By selective sweep, the mutation conferring resistance to the antimalarial drug is favoured and thus increases in frequency in the population (*i.e.*, “sweeps“ through the parasite population) while the linked neutral variation in the markers flanking the advantageous allele are removed (Peterson *et al.*, 1991, Gyang *et al.*, 1992, Plowe *et al.*, 1995, Mc Collum *et al.*, 2008). *Pfdhfr/pfdhps* variants have emerged in African parasite populations under SP to confer resistance to SP by impairing enzymes’ function in a synergistic manner (Eldin *et al.*, 1995, Pearce *et al.*, 2005). Further selective sweeps of highly resistant *dhfr* alleles have been described for samples from the Thailand/Myanmar border and from South America (Nair *et al.*, 2003, McCollum *et al.*, 2007)

Point mutations at *pfdhfr* codons 51, 59, 108, and 164 act synergistically to increase resistance to pyrimethamine. Specifically, the S108N mutant has a low level of resistance or tolerance, the N51I-S108N and C59R-S108N double mutants have moderate levels of resistance, the N51I-C59R-S108N triple mutant has a higher level, and the N51I-C59R-S108N-I164L quadruple mutant parasite is considered to be fully resistant to the effects of pyrimethamine (Cortese & Plowe, 1998, Plowe *et al.*, 1998). Similarly, mutations at *dhps* codons 436, 437, 540, 581, and 613 act synergistically to increase the level of resistance to sulfadoxine. Simply, the mutations S436A and A437G alone confer a low level of resistance, and when these are in a combination with K540E, A581G, and/or A613S, the parasite has an increased level of resistance to sulfadoxine (Gregson & Plowe, 2005, Triglia *et al.*, 1998). For

instance, in East Africa, the *pfdhfr* triple mutation, *pfdhps* A437G-K540E and their combination strongly reflected severe SP pressure and predicted SP treatment failure (Kublin *et al.*, 2002).

Based on these observations, the prevalence of *pfdhfr* double C59R-S108N and of *pfdhps* A437G and K540E reported in the present study suggests SP resistance to be pronounced in the study area but not yet highly intense (Gregson & Plowe, 2005).

As compared to recent molecular data from India, the observed *pfdhfr* double mutation C59R-S108N (*i.e.*, without N51I) is found rather in central India (Patel *et al.*, 2017, Pathak *et al.*, 2014) whereas *pfdhfr* triple mutations (C59R-S108N *plus* N51I or I164L) have become prevalent particularly in north-eastern India (Mohapatra *et al.*, 2014, Mishra *et al.*, 2014, Sharma *et al.*, 2015). Likewise, the *pfdhps* mutations A437G and K540E and the respective *pfdhfr/pfdhps* combinations, are comparatively rare in central India but common in the Northeast.

Our data from south-western India occupy a middle position in this regard: while *pfdhfr* C59R-S108N occurs at a prevalence similar to central India, the *pfdhps* mutations A437G and K540E are almost as common as in north-eastern India, where the high levels of SP resistance reported have led to a change in current drug policies (Patel *et al.,* 2017, Sharma *et al.*, 2015).

We detected *pfdhfr* C59R-S108N *plus pfdhps* A437G or *plus* A437G-K540E in almost two in three isolates. Although double *pfdhfr* mutations do not greatly intensify resistance towards SP in the parasite population, in Indonesia SP treatment failure was observed in patients infected with parasites harbouring *pfdhfr* C59R-S108N *plus pfdhps* A437G, while *pfdhfr* double - *pfdhps* double variant was associated with high grade of SP resistance (Nagesha *et al.*, 2001).

Despite the limited data set, SP resistance in Mangaluru seems to be a local rather than an imported problem. The prevalence of *P. falciparum* mono-infection was significantly increased in construction workers and reduced in migrants from north/north-eastern India, although both factors overlapped (Gai *et al.*, 2018). These could be due to a higher degree of *P. falciparum*-related semi-immunity in migrants from north-eastern Indian states as compared to the local population from Mangaluru and consequently some degree of resistance to locally transmitted *P. falciparum* parasites.

Given ongoing SP drug pressure, *e.g.*, on parasites transmitted to recently treated patients without detectable artesunate levels but fading SP concentrations, and the foreseeable, stepwise development of further *pfdhfr/pfdhps* mutations (Mita *et al.*, 2014) SP resistance is likely to intensify in the study area, eventually compromising AS+SP.

Furthermore, to protect the artemisinin component, partner drugs should have the highest possible efficacy. The India National Drug Policy on malaria recommends the use of artemether-lumefantrine (AL) in the north-eastern states (NVBDCP, 2013) which bear intense SP resistance.

In the present study, the *pfmdr1* haplotype N86-184F-D1246 (NFD) was detected in 98.2% of isolates screened. The predominance of the *pfmdr1* N86 allele and the almost fixation of the NFD alleles combination indicate that artemether-lumefantrine (AL) might not be a promising candidate for replacing AS+SP in this area. In fact, *in vitro* studies showed that *P. falciparum* parasites harboring the *pfmdr1* wildtype allele N86 have reduced sensitivity to DHA and to ACT's partner drugs lumefantrine and mefloquine (showing 3-4-fold higher half maximal inhibitory concentration values) but increased susceptibility to CQ, to the active metabolite of amodiaquine (AQ) and, less pronounced, to piperaquine (PPQ) (Veiga *et al.*, 2016).

In addition, data have provided clear evidence of a post-AL treatment selection of *pfmdr1* alleles such as N86 and 184F associated with a significant decrease in lumefantrine susceptibility (Sisowath *et al.*, 2007; Malmberg *et al.*, 2013). Similarly, *pfmdr1* NFD parasites re-infecting after AL treatment have been shown to tolerate 15-fold higher AL blood concentrations than those with the opposite YYY haplotype even though the central polymorphisms appeared to be *pfmdr1* N86 (Malmberg *et al.*, 2013, Venkatesan *et al.*, 2014).

Interestingly, in a study conducted in Tanzania, a decreased prevalence of *pfmdr1* mutant alleles 86Y and 1246Y, and wildtype Y184 were linked to the extensive and prolonged use of AS+AQ (Fröberg *et al.*, 2012). Further studies pointed out that *P. falciparum* isolates carrying *pfmdr1* mutated 86Y and Y184 wild alleles were found to be associated with the extensive usage of AQ and CQ in time (Humphreys *et al.*, 2007; Dlamini *et al.*, 2010; Mungthin *et al.*, 2010; Thomsen *et al.*, 2011).

According to these considerations, AL and AQ select different *pfmdr1* alleles, which suggests that AS+AQ and DHA+PPQ might be effective in parasites with reduced susceptibility to AL (Venkatesan *et al.*, 2014, Blasco *et al.*, 2017, Haldar e*t al.,* 2018). In addition, the variation over time of the prevalent *pfmdr1* haplotype in an area seems to highly reflect changes in the antimalarials used since *pfmdr1* mutated allele 86Y and wildtype allele Y184 changed to a haplotype carrying wildtype N86 and mutated 184F alleles years after the implementation of ACT.

Taken together, in the event of SP treatment failure, ACT combinations such as AS+AQ and DHA+PPQ seems more recommendable than AL in the study area as well as the potential re-introduction of CQ. Furthermore, in the event of an ACT replacement *i.e.*, in absence of SP pressure, *pfdhfr* and *pfdhps* mutations may confer a disadvantage to the parasite, allowing them to be outcompeted by susceptible parasites with fully functional PfDHFR and PfDHPS enzymes (Plowe *et al.*, 1996, Curtis *et al.*, 1996).

For what concerns ART resistance, recent studies have reported *K13*-propeller mutations in southeast Asian countries (Ménard *et al.*, 2016) and in Bangladesh (Mohon *et al.*, 2014). However, in India, very limited polymorphisms have been detected so far (Bharti *et al.*, 2016, Mishra *et al.*, 2016, Das *et al.*, 2018). In the present study, all samples were found

to be wildtype, which agrees with previous reports from the West Bengal region and from central India (Chatterjee *et al.*, 2015, Patel *et al.*, 2017).

However, against the background of evidence for impaired, and potentially further waning SP efficacy, *K13* mutations may emerge or spread after importation. In this regard, a limited number of *K13* mutations have recently been detected in the north-eastern state of Arunachal Pradesh bordering Myanmar (Mishra *et al.*, 2016).

The diversity of malaria in India, including geographically variable drug susceptibility and resistance alleles, impedes drug policy recommendations, which accurately fit for the whole of the sub-continent. India currently forms the western boundary of ART-resistant malaria, and the term of the currently employed first-line treatment AS+SP is limited. Against the background of spreading and intensifying SP resistance as seen in the present study, expanded monitoring of molecular makers as well as clinical trials on alternative first-line antimalarials are required.

4.3. Detection of putative *pvmdr1* mutations associated to CQ resistance in *P. vivax* isolates from Mangaluru, India, in 2015

Chloroquine (CQ) resistance in *Plasmodium vivax* is becoming more and more widespread, hindering management of clinical cases and posing a huge threat to the health of millions of people exposed to the risk of vivax malaria.

Compared to falciparum malaria, drug resistance studies on *P. vivax* that focus on epidemiology, drug efficacy and mechanisms of resistance are rare. Retrospective and prospective analysis of *in vivo* therapeutic efficacy studies (TES) of CQ are the golden standard to detect clinical failures but are limited by *P. vivax* recurrent parasitaemia cases. Moreover, the lack of a standardized culture system limits the usefulness of *in vitro* susceptibility tests for detection of drug resistance (WHO, 2015).

Although there are no validated molecular markers for drug-resistant vivax malaria, potential candidates were reported, most being homologues of drug resistance makers of *P. falciparum*, such as *pvmdr1* (Rieckmann *et al.*, 1989, White, 2002, Baird, 2013).

In the current study, we showed the presence of ten mutant haplotypes in *P. vivax* molecular marker *pvmdr1* among 108 isolates collected in the costal city of Mangaluru, India, in 2015. Eight non-synonymous polymorphisms were detected including mutations T958M and F1076L. These mutations were present in all and in 87% of the genotyped samples, respectively. Furthermore, the substitution Y976F was observed in one isolate in combination with S513R-T958M-F1076L mutant alleles.

There are a number of contradicting reports regarding the association between these *pvmdr1* polymorphisms and CQ resistance. Few studies associated Y976F mutation in *pvmdr1* with an increase in the CQ half maximal inhibitory concentration (IC_{50}) value of *P. vivax* isolates *in vitro* (Suwanarusk *et al.*, 2007, Lu *et al.*, 2011). Amino acid substitutions Y976F and F1076L have been detected in Thailand and in Indonesia where CQ resistance has been reported for over a decade (Brega *et al.,* 2005). Morevoer, the detection of one isolate carrying the Y976F mutation in Rajasthan, India, was suggested to indicate the beginning of a trend towards decreased CQ sensitivity in the area (Garg *et al.,* 2012). At last, in a study conducted in Madagascar, treatment failure was observed in 4 patients carrying the *pvmdr1* sextuple mutant S513R-698S-908L-T958M-Y976F-F1076L (Barnadas *et al.*, 2008).

However, other *in vitro* studies have suggested that Y976F mutation reduces susceptibility to mefloquine (MQ), while increasing susceptibility to CQ. In one report from Suwanarusk and co-workers, cryopreserved Thai isolates harbouring the *pvmdr1* Y976F mutation showed higher susceptibility to CQ *in vitro* as compared to *P. vivax* wildtype isolates (Suwanarusk *et al*., 2008).

Other studies have shown that the prevalence of mutations in *pvmdr1* is higher in areas with current or past intense use of MQ such as in French Guiana and Cambodia, than it is in regions where it has never been used hence the extended usage of this drug may select for mutant *pvmdr1* haplotypes (Khim *et al.*, 2014). In India, MQ has been employed for over two decades for chemoprophylaxis and for treatment of CQ-resistant *P. falciparum* parasites, and has been considered as a component of ACT in combination with artesunate (Valencha *et al.,* 2013, Anvikar *et al.,* 2014).

The high prevalence of *pvmdr1* T958M and F1076L in our study is in accordance with the genotype pattern previously reported in India and at this location (Lu *et al.*, 2011, Shalini *et al.*, 2014). In fact, in a previous study performed by Joy and colleagues on 85 *P. vivax* isolates from Mangaluru, mutations T958M and F1076L were detected in 90% and 76%, respectively. However, while the candidate marker Y976F occurred only once (0.9%) in the present study, the figure was almost 8-fold higher in their report (Joy *et al.*, 2018). Moreover, 15 isolates collected at Kasturba Medical College in Mangaluru in 2016 were carrying the mutation T958M (100%) and F1076L (14/15, 94%) (Anantabotla *et al.*, 2019).

In our study, 89.5% of vivax malaria patients cleared parasitemia within two to three days of CQ treatment, *i.e.*, in 88% and 94% of patients on Day-2 and Day-3, respectively. In a meta-analysis of *P. vivax* CQ resistance, the earliest treatment failure occurred at a median of 14 days (range: 3–28), and early parasite clearance correlated with treatment outcome as assessed on Day-28 (Price *et al*., 2014). Of note, parasite clearance in 95% or 100% of patients by Day-2 or Day-3, respectively, was found to be 100% predictive of CQ sensitivity as defined by Day-28 outcome. The present study was not designed as a treatment trial but against this background, it seems justifiable to state that CQ in the study area is sufficiently effective. In addition, none of the *pvmdr1* polymorphisms was associated with early parasite clearance.

Sufficient CQ efficacy in the study area is supported by the virtual absence of the candidate CQ resistance marker *pvmdr1* Y976F, the lacking association of the detected polymorphisms with follow-up positivity and the absence of sextuple *pvmdr1* mutants carrying mutation S513R and Y976F as observed in treatment failures reported in Madagascar (Barnadas *et al.,* 2008).

In a recent study conducted at four different tertiary care hospitals from distinct geographical regions of India, including Karnataka (Anantabotla *et al.*, 2019), the abundance of the *pvmdr1* T958M mutation followed by F1076L was regarded as a sign of high predominance of CQ-resistant *P. vivax* isolates. Certainly, the role of these mutations in clinical CQ resistance needs to be further evaluated in patients infected with mutant variants. Unfortunately, assessment of the therapeutic failures is confounded by the tendency of *P. vivax* malaria to relapse from liver hypnozoites several weeks after initial infection, and especially in endemic areas of high transmission, it is hard to distinguish between re-infection,

relapse, and recrudescence (Whitby, 1997, Pukrittayakamee *et al.*, 2004, Sattabongkot *et al.,* 2004). Furthermore, as for *P. falciparum*, drug susceptibility tends to be stage-specific, with significantly higher IC_{50} values in trophozoites as compared to ring-stage parasites upon exposure to CQ (Russel *et al*., 2008).

Thus, we are still unable to assume that these polymorphisms could be related to any drug resistance, and the same polymorphisms have not been described yet for other Plasmodium species such as *P. berghei, P. chabaudi,* and *P. yoelii (*Brega *et al.,* 2005).

However, as with most previous investigations, the present data do not support a predictive role of these polymorphisms in CQ resistance. Ultimately, prolonged monitoring of treated patients is required to elucidate the role of *pvmdr1* variants in recrudescence and to enable the prompt detection of CQ resistance in south-western India.

4.4. Prevalence of *pfdhfr/pfdhps* quadruple mutant but absence of SP super-resistance among asymptomatic pregant women following IPTp in Calabar, Nigeria, in 2013-2014

In this study conducted in Calabar, Nigeria on 28 asymptomatic pregnant women before the first dose of IPTp, molecular analysis revealed a very high prevalence of *pfdhfr* triple mutant N51I-C59R-S108N. This haplotype was almost fixed in the study sample with a frequency of 93%. No *pfdhfr* I164L mutation was detected, a finding in accordance with a previous report from Nigeria (Happi *et al.*, 2005).

The *pfdhfr* triple mutation N51I-C59R-S108N is known to confer intense pyrimethamine resistance *in vitro* (Gregson & Plowe 2005). Higher SP resistance is associated with an increasing number of mutations in *pfdhfr* gene followed by mutations in the *pfdhps* gene. Infections bearing triple mutant *pfdhfr* (N51I-C59R-S108N) have high treatment failure rates, and quadruple mutant *pfdhfr* alleles (N51I-C59R-S108N-I164L) render parasites untreatable (Plowe *et al.,* 1997). Every successive mutation in both genes incrementally increases the parasite tolerance to SP *in vitro* (Wu *et al.,* 1996, Triglia *et al.,* 1997, Triglia *et al.,* 1998).

In Africa, *pfdhfr* mutations have emerged more than a decade before the *pfdhps* mutant genotype and are now well established across sub-Saharan Africa (Talisuna *et al*., 2004). To varying degrees, *pfdhfr/pfdhps* mutations are prevalent throughout all Africa, and the increasing failure rate of SP treatment in children has rendered SP unsuitable for therapy. Especially in southern and eastern Africa, the escalation of SP resistance coincided with the emergence of double mutant *pfdhps* alleles in parasite populations already harbouring *pfdhfr* triple-variants. This so-called *pfdhfr*/*pfdhps* “quintuple mutant” haplotype which is a combination of a triple *pfdhfr* mutation (N51I-C59R-S108N) and double *pfdhps* mutation (A437G-K540E) is predictive of SP treatment failure (Duraisingh *et al.,* 1998, Happi *et al.,* 2005, Vinayak *et al.,* 2010). A study performed by Triglia *et al*. in 1998 pointed out that the *pfhdps* double mutant A437G-K540E can raise sulfadoxine tolerance up to 200-fold, compared with just ten-fold for the A437G substitution alone (Triglia *et al.*, 1998).

The appearance of *pfdhps* double mutants on a background of pre-existing triple *pfdhfr*-resistant variants has been observed in Kenya in 1993-1995, where pyrimethamine resistance was present since 1988 (Mberu *et al.,* 2000, Nzila *et al.*, 2000), in Tanzania in 1995, in Malawi in 1995-1996 and in South Africa in 1999-2000 (Curtis *et al.,* 1996, Plowe *et al.*, 1997, Roper *et al.,* 2003). A Nigerian study (Happi *et al.,* 2005) reported a strong association between the quintuple mutant haplotype and SP treatment failure in children less than five years old.

The high level of *pfdhfr* triple mutant parasites observed in the current study is in accordance with these previous data and could reflect the extended use of pyrimethamine in the past for weekly chemoprophylaxis to prevent malaria in pregnancy (Fawole & Onyeaso 2008; Yusuf *et al.,* 2008). Furthermore, cotrimoxazole use, *e.g.*, for the prevention of *Pneumocystis jirovecii* in HIV infected individuals, has been associated with the emergence, spread and intensification of the A437G and K540E mutations in the *pfdhps* gene (Gesase *et al.,* 2009). In Nigeria, there is a high burden of pneumonia, and cotrimoxazole is commonly used for treatment among HIV patients and children with pneumonia (Onyedum & Chukwuka, 2011).

In the *pfdhps* gene, the frequency of the core mutation, A437G, was over 90%. All *pfdhps* haplotypes containing A437G are known to confer resistance to sulfadoxine *in vitro* (Triglia *et al*., 1998). The combination of *pfdhps* A437G with the *pfdhfr* triple mutation N51I-C59R-S108N is considered to be associated with SP treatment failure (Mockenhaupt *et al.,* 2005) and was here detected in over 70% of the isolates analysed.

Although several studies report mutant A437G to be very frequently found in association with the substitution K540E (Kublin *et al.,* 2002), this pattern tends to be less common in West Africa (Pearce *et al.,* 2009). In our study, *pfdhps* K540E was absent from all *P. falciparum* isolates investigated. Thus, there was no *pfdhps* double mutation at codon 437 and 540 that, when combined with *pfdhfr* triple mutation N51I-C59R-S108N, undermines the ability of SP to clear existing *P. falciparum* infections in asymptomatic pregnant women (Kalilani *et al*., 2007, Desai *et al*., 2016), and shortens post-treatment prophylactic period following IPTp (Kublin *et al.,* 2002; Plowe *et al.,* 2004).

Despite the scarce detection of *pfdhfr/pfdhps* quintuple mutants in western African countries, few studies have reported the emergence of the K540E mutation. In fact, the A437G-K540E haplotype has been reported at low frequencies in samples collected in Guinea in 2004–2005 (7%) and in Ghana in 2003 and 2005 (1%) (Pearce *et al.*, 2009). Furthermore, few studies from the western part of Nigeria have found an emergence of mutant *P. falciparum* isolates carrying sulfadoxine resistance associated to A437G and K540E mutations in the *pfdhps* gene (Happi *et al.,* 2005, Olasehinde *et al.,* 2014, Iwalokun *et al.,* 2015). Furthermore, the co-occurrence of A437G with A581G has been considered to confer a certain level of SP resistance (Pearce *et al.,* 2009). In the current study, *pfdhps* A437G-A581G was present in 20 of 28 isolates.

Compared with other markers of SP resistance, fewer data are currently available on the distribution of the *pfdhps* mutation A581G and its impact on parasite survival rates and fetal growth. Five studies performed in eastern and southern Africa reported substantial heterogeneity in the number of low birthweight events observed in pregnant women infected with parasites harbouring *pfdhps* mutation A581G that were receiving IPT with SP. In the smaller studies, with only 4-8 low birthweight events in the reference groups, a pooled effect size of 35% was observed (Harrington *et al.,* 2011, Minja *et al.*, 2013, Braun *et al.*, 2015),

whereas in the two more recent studies with larger reference groups the average effect size was of −2% (Chico *et al.*, 2017, Desai *et al.*, 2018).Nevertheless, in a recent wide analysis of survey data performed on a much larger sample size recruited in areas with a high prevalence of *pfdhps* mutation A581G (of approx. 35%), no effect on low birthweight was reported (van Eijk *et al.*, 2019). Importantly, these studies were performed in areas where the mutation K540E was also prevalent.

In the present work, the *pfdhfr/pfdhps* mutant N51I-C59R-S108N/A437G-A581G-K540E was not detected. This so-called “sextuple mutant” is associated with enhanced SP resistance *in vitro*, increased risk of therapeutic failure in patients with acute malaria (Triglia *et al.*, 1998, Gregson *et al.*, 2005, Picot *et al.*, 2009), placental inflammation and decreased infant birth weight in pregnant women following IPT-SP, and increased parasite growth in presence of SP (Harrington *et al.*, 2009, Harrington *et al.*, 2011, Eisele *et al.*, 2012). This latter finding, if broadly documented, would suggest that SP exacerbates placental pathology in areas with highly drug-resistant *P. falciparum* parasites.

In fact, in high-resistance areas, inter-strain competition in polyclonal infections may have selected for parasites harbouring the additional mutation at codon 581 as these parasites with high grade of SP resistance have a survival advantage under drug pressure over less resistant parasites and may outcompete them, ultimately leading to better survival and growth than in the absence of SP (Harrington *et al.*, 2009). Nevertheless, further studies suggested that although SP use is of decreased benefit in the presence of the sextuple mutant, there was no evidence that its use was harmful *per se* (Minja *et al.*, 2013, Gutmann *et al.*, 2015).

To date, the sextuple mutant has been identified at a rate of over 10% in only two areas *i.e.*, in north-eastern Tanzania, and at the crossing borders of south-western Uganda, eastern Rwanda, eastern Democratic Republic of the Congo and north-western Tanzania (van Eijk *et al.*, 2019).

Finally, the prevalence of *pfdhps* S436A, which is an additional mutation that generally follows the emergence of A437G, was here over 80% of the isolates. This additional mutation corresponds to an increase in the degree of resistance to SP as well. Flanking sequence analysis revealed that the mutation S436A is the result of only recent selection due to SP pressure and it has a highly distinctive regional geographic distribution (Pearce *et al.*, 2009).

Taken altogether, *pfdhfr* and *pfdhps* gene mutations associated with SP resistance appeared to be highly prevalent among asymptomatic pregnant women in the current study area but because of the small sample size of the study, further investigations should be conducted. The prevalence of the triple *pfdhfr* mutation and the double *pfdhps* A437G-A581G mutation were very high suggesting that the efficacy of SP as IPTp in southeast Nigeria might be severely threatened. Nevertheless, the role played by mutation A581G remains uncertain as its impact on low birthweight seems to variate according to the sample size, causing discrepancies in studies’s results.

Also, although the effectiveness of IPTp in preventing low birthweight events decreases with increasing SP resistance, IPTp with SP remains associated with a 7–10% reduced risk of low birthweight outcome even in areas where resistant quintuple mutant haplotypes are fixed (van Eijk *et al.*, 2019). This small but persistent effect on low birthweight contrasts with the incapability of SP to eradicate malaria infection and with the unfavourable parasitological response observed in asymptomatic pregnant women receiving IPTp-SP in high-resistance areas (Desai *et al*., 2016).

That IPTp with SP can decrease the risk of low birthweight even in areas where its efficacy for clearance of infection is compromised might suggest that suppression, rather than radical clearance of parasites, is required to mitigate the adverse effects of malaria on placental function and fetal growth, as observed in multigravidae (who acquire protective antimalarial immunity over successive pregnancies). Alternatively, SP might have beneficial effects on birthweight that are independent of its antimalarial properties hence that are not affected by parasite resistance such as antimicrobial activity or immunomodulatory effects similar to those described for cotrimoxazole (Capan *et al.*, 2010, Church *et al.*, 2015, Chico *et al.*, 2017, Desai *et al.*, 2018).

Of note, in areas where the prevalence of quintuple mutants is more than 50%, the prevalence of *dhps* mutation A581G serves as a proxy for the so-called sextuple mutant (van Eijk *et al.*, 2019). However, the differences observed in the combination of the resistance alleles at *pfdhps* codons in *P. falciparum* parasite populations from East and West Africa underline their distinct geographical origins (Pearce *et al.*, 2009).

In a recent work published by van Eijk and colleagues, the co-occurrence of *pfdhps* mutations K540E and A581G was suggested to predict the intensification of SP resistance in eastern and southern Africa (Figure 4.2) while in central and western Africa, where *pfdhps* K540E mutation is absent or rare, tracking *pfdhps* A437G might be purely informative (van Eijk *et al.*, 2019).

Furthermore, other mutations have started to emerge in West Africa, such as *dhps* I431V, which has been reported on a haplotype bearing mutant alleles at codons 581 and 613 but a wild-type allele at codon 540 (Chauvin *et al.*, 2015, Oguike *et al.*, 2016). The clinical implications of such new haplotypes require further study.

In the light of these considerations, the *pfdhfr/pfdhps* alleles pattern reflecting the intensification of SP resistance in West Africa is still unclear. The absence of *pfdhps* K540E mutation in the present study may suggest that SP is still efficacious when used as IPTp but each new mutations occurring in *pfdhfr/pfdhps* markers at this location will be equally relevant and will therefore need to be further investigated. Nevertheless, screening for *pfdhps* K540E remains a priority in Nigeria and West Africa in order to avoid the spreading of the sextuple mutant westwide. Also, although the World Health Organization still recommend IPTp-SP administration in areas where a high proportion of *P. falciparum* parasites carry quintuple

pfdhfr/pfdhps mutations (ter Kuile *et al.*, 2007, WHO, 2014), the evaluation of alternative preventive treatment options for malaria chemoprophylaxis in pregnancy may be necessary.

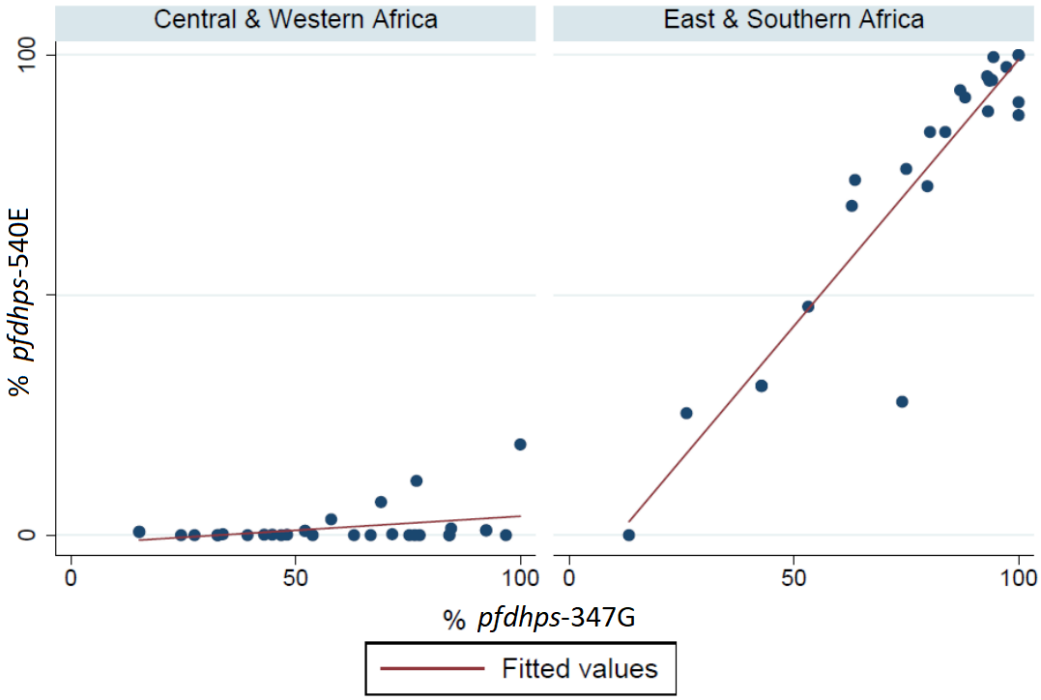

Figure 4.2: Relationship between the prevalence of the *pfdhps* mutations A437G and K540E in central and western Africa (slope of approx. 0.06) **and in eastern and southern Africa** (slope = 1.1). While the occurrence of *pfdhps* A437G and K540E do not relate in central and western Africa, they co-occurred in eastern and southern Africa, where *pfdhps* mutation A437G is considered a predictor of the *pfdhfr/pfdhps* sextuple mutant N51I-C59R-S108N/A437G-A581G-K540E. Figure adapted from van Eijk *et al.*, 2019.

4.5. High *ex vivo* RSA survival rates and Day-3 positivity after ACT in *P. falciparum* patients from Huye, southern Rwanda, 2018

Between March and May 2018, an observational study on ART resistance was carried out in the southern province of Rwanda. To assess the prevalence of ART-resistant parasites in the area, the survival rates of *P. falciparum* isolates freshly collected from malaria patients were evaluated by *ex vivo* ring-stage survival assay (RSA). Among the 214 isolates screened, 16.4% were considered suitable for the assay and processed. Survival rates greater than 10% were observed in 9 isolates, one of which coincided with a slow clearance infection *in vivo.* This study pointed out the potential emergence of a parasite population with reduced susceptibility to ART in the Huye district, southern province of Rwanda.

Ex vivo RSA is considered a reliable tool to assess ring-stage specific reduction of ART susceptibility in SEA and a valid alternative when no daily counts of parasite density during treatment can be performed (*e.g.,* in OPD patients) (Phyo *et al.*, 2012, Ashley *et al.*, 2014). Its correlation with delayed parasite clearance *in vivo* was firstly assessed in Cambodian *P. falciparum* isolates collected from patients with PCHL >5 h after ACT administration that presented *ex vivo* RSA survival rates >10% (Witkowski *et al.*, 2013, section 1.5.2.).

In Africa, few attempts have been made to detect ART-resistant parasites by using *ex vivo* RSA. In two reports from Cameron and Uganda (Kampala), *ex vivo* RSA was performed on 64 and 43 isolates, respectively, but no isolate had survival rates equal or greater than 10% and all patients successfully recovered after 3 days of ACT regimens (Cooper *et al.*, 2015, Ménard *et al.*, 2016). In a recent study performed in Gulu, northern Uganda, 194 *P. falciparum* isolates collected between 2014 and 2016 were processed by *ex vivo* RSA. Among these, 4 (2.1%) were classified as having high parasite survival rates ranging from 13.3% to 34.3%. One isolate had higher survival rates than ART-resistant laboratory-adapted parasite clones obtained from Cambodian lines (Ikeda *et al.*, 2018).

The *ex vivo* RSA results obtained in the current study seem to support the hypothesis raised by Ikeda and co-workers of an emergence of an ART-resistant parasite population in sub-Saharan Africa. One in four isolates processed with *ex vivo* RSA showed survival rates greater than 10%, two of which exhibed the highest *ex vivo* RSA survival rates ever reported in Africa, *i.e.,* 38% and 47%. Furthermore, parasite isolates collected in April and May 2018 where overall more resistant (higher number of samples with high survival rates) to DHA exposure than those processed in March, most likely reflecting a greater genetic variation occurring with the progress of the rainy season.

However, only one isolate with survival rates of 20% at RSA was associated with a slowly clearing infection *in vivo.* This observation may reflect the multi-faceted interconnection

between host and pathogen occurring in high transmission areas in sub-Saharan Africa. As discussed in section 1.3.2., semi-immunity considerably reduces the parasitemia load, slowing down the emergence of *in vivo* resistance. Spontaneously generated drug-resistant parasites must deal not only with the antimalarial drug concentrations but also with the major anti-parasitic effect of host defence and this further reduces the individual parasite survival probability. Even if the resistant parasite does survive the initial drug treatment, and multiplies, the chance that this will result in sufficient gametocytes for transmission is likely to be reduced (White, 1999). Thus, it does not come with surprise that an infection with *P. falciparum* parasites able to survive clinically relevant concentrations of ART may still be efficiently cleared by the patient immune system.

Nevertheless, our study has a few important limitations. Firstly, the developmental stages of the parasites at enrolment were not defined, *i.e.,* no differentiation between early ring-stages and late ring-stages parasites was done. Parasites that exhibit high survival rates in the *ex vivo* RSA transcriptionally arrest at their ring-stage following exposure to the drug, entering a state of dormancy that coincides with the condensation of their cytoplasm (Tucker *et al.*, 2012). After DHA removal, dormant parasites rapidly resume growth and proceed through the erythrocytic cycle producing numerous parasites able to enter drug-exposed dormancy. The ability to become dormant and to awake following drug exposure is limited to a very narrow amount of early ring-stage parasites (Klonis *et al.*, 2013, Witkowski *et al.*, 2013, Witkowski *et al.*, 2013b) and appears to impact artemisinin resistance *in vitro* (Teuscher *et al.*, 2012, Cheng *et al.*, 2012). Since in our assay all stages have been processed, this may have altered *ex vivo* RSA outcome in those isolates where older parasites were predominant as being susceptible rather than resistant.

Secondly, patients were asked to return on Day-3 of ACT regimen for parasite clearance assessment, that is, only the first dose of treatment was taken under observation. Since no measurement of drug concentrations in blood or plasma was performed upon patient's return to the hospital, no clear distinction between true clinical resistance and inadequate drug exposure could be assured.

Thirdly, *Plasmodium* species were ascertained only by light microscopy before *ex vivo* RSA performance according to WWARN-approved guidelines (Witkowski *et al.*, 2013). Therefore, putative sub-microscopic inter-strain or inter-species infections, which occur at high frequency in Africa, were not excluded from the assay. The presence of other parasite genotypes competing with the resistant parasites for erythrocyte infection may have further reduced the survival rates of the latter ones both *ex vivo* and *in vivo* since most mutants have reduced fitness as compared to wildtypes (Dye & Williams, 1997, Mayxay *et al.*, 2004, McKenzie *et al.*, 2006). Given this premise, patients may have cleared efficiently the infection in spite of parasite drug resistance or kept the infection at a sub-microscopic level.

Sub-microscopic infections are extremely worrisome since surviving parasites can propagate leading to a recrudescent infection (White, 1997). In a study conducted in Uganda in 2016 by Chang and colleagues, the genotyping of 64 samples revealed the presence of a low level reservoir of circulating ring-stage parasites 14 days after the first administration of AL, indicating that these parasites persisted after a fully observed curative dose of ACT (Chang *et al.*, 2016). In Africa, when no molecular analysis can be performed, recrudescence studies are made difficult by the high frequency of vector biting, semi-immunity and premunition conditions. For this reason and because of limited funds, in this study no parasite count was performed on Day-14 or Day-28 after ACT to assess recrudescence.

Furthermore, the significance of *ex vivo* RSA data are undermined by the fact that there is hardly any evidence of the correlation between high RSA survival rates, *K13* mutations and ART-resistant phenotype (intended as PCHL >5 hours) in *P. falciparum* isolates of African origin.

In 2017, Lu and colleagues reported the first case of indigenous ART-resistant African isolate in a Chinese patient returning from Equatorial Guinea. Although after ACT treatment the patient still had detectable parasitemia in his peripheral blood and the parasite was shown to harbor a novel *K13* mutation (mutation M591I, section 4.1), the isolate had an *ex vivo* RSA survival rate of approx. 2.3% after exposure to clinically relevant concentrations of DHA, far below the level associated with ART-resistant strains (Lut *et al.*, 2017) (Figure 4.3).

These data cast serious doubts on the overall significance of genotype analysis and *ex vivo* assays to define parasite clarance rates *in vivo* in the African continent.

As discussed in section 4.1, in low-transmission regions (particularly in Asia), the majority of malaria cases are symptomatic, therefore selection takes place in the context of treatment where a relatively large number of parasites in a non-immune individual encounter the antimalarial drugs. In areas with intense malaria transmission, most infections are asymptomatic and these are acquired repeatedly throughout life. People still receive antimalarial treatments as in low-transmission settings but these ‘treatments’ are largely unrelated to the peaks of parasitaemia, thereby reducing the probability of an effective selection for resistance (White, 2002). Moreover, in spite of the increased likelihood that a feeding *Anopheles* will encounter a resistance-bearing *P. falciparum* gametocyte, resistant parasites developed in the mosquito must still be transmitted to a susceptible recipient for resistance to spread.

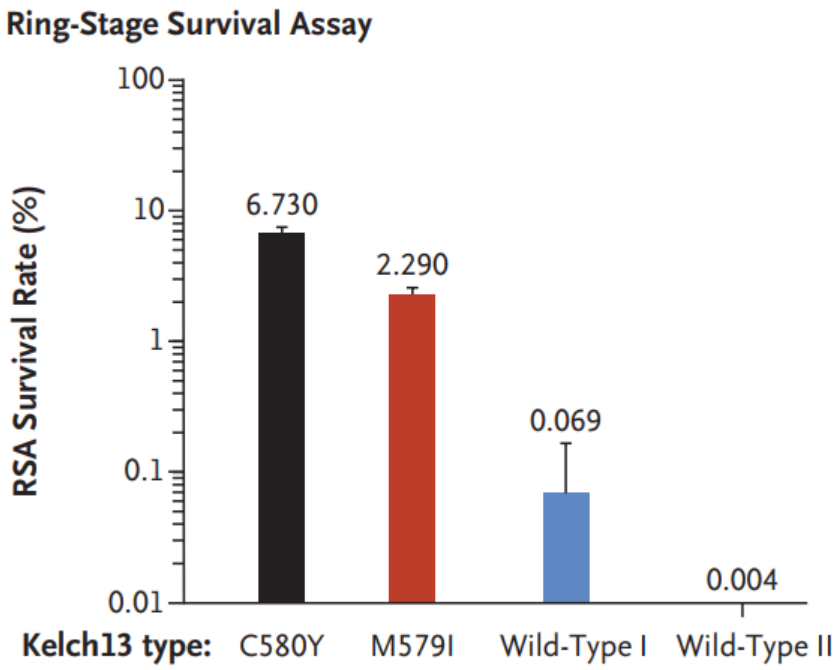

Figure 4.3: Relationship between *ex vivo* RSA survival rate and *K13* mutation in 4 isolates collected in China from patients returning from Equatorial Guinea.
Two patients carrying the validated *K13* mutation 580Y and the novel mutation 591I had survival rates under 10%. Figure adapted from Lu *et al.*, 2017.

According to previous works (Miotto *et al.*, 2013, Takala-Harrison *et al.*, 2015, Amato *et al.*, 2016) ART resistance does not originate from a single determined *K13* polymorphism but rather as consequence of multiple independent *K13* polymorphisms emerging at low frequencies in individual patients from the same geographic region (Talundzic *et al.*, 2017). The identification of the previously reported substitution P574L (section 4.1.) in only one isolate may indicate that this is a minor allele whose variants are transient and may subsequently be lost if there is a fitness cost. As indicated by previous work in the MalariaGEN project (Amato *et al.*, 2016), the parasite population in Africa is rich in low-frequency alleles, suggesting that the majority of these substitutions are not uniformly present in entire lineages or sublineages. Thus, this mutation may be neutral and unique to this parasite population.

Furthermore, as the majority of the Rwandan population is semi-immune, the individual probability of resistance propagation is likely to be reduced. In high-transmission areas only young children (<5 years) develop significant symptoms reaching high parasite densities. In Rwanda, only 14.6% of the population is represented by children under 5 years of age that contribute to the scenario of *de novo* selection of resistance (with blood volumes in infants 5–20 times lower than in adults, and therefore fewer total parasites for any given density) (PMI, 2015). Nevertheless, clearance times in young children in high-transmission areas following antimalarial drug treatment are nearly always faster than corresponding values in non-immune children and adults in low-transmission areas (White & Pongtavornpinyo, 2003).

Given these considerations, the mutations in *K13* observed in Africa (including P574L and C469F alleles) could be part of a neutrally evolving population or in which case they reflect the emergence of a resistance, the latter would be masked by a favorable clinical outcome since the presence of semi-immunity in the African population favours prompt recovery after treatment also when the parasites have reduced sensitivity towards the administered drugs (Rogerson *et al.*, 2010, Lopera-Mesa *et al.*, 2013).

It is important to remember that the standard for monitoring the emergence of ART resistance remains the assessment of ACT in therapeutic efficacy studies. A recent clinical trial performed in the eastern province of Rwanda reported late clinical failure after AL treatment in 1.7% patients on Day-28 and 2.3% on Day-42 (Uwimana *et al.*, 2019). This low treatment failure rate suggests that AL is still highly efficacious in Rwanda a decade after its introduction as first-line treatment for uncomplicated *P. falciparum* malaria and that the recent malaria resurgence documented throughout the country most likely does not come from reduced AL efficacy.

Understanding how *K13* patterns are emerging due to drug pressure and their correlation to *ex vivo* RSA and i*n vivo* outcomes is critical for monitoring artemisinin resistance in Africa. As malaria control strategies intensify and countries enter the preelimination phase, continued surveillance to identify trends that may warrant further investigation and prioritize *K13* alleles for functional testing and validation is highly required. Knowing these important patterns and predicting inflection points in the African continent will be critical for the continued success in reducing malaria burden.

5. Conclusions

Malaria elimination and control is a global health challenge and a major public health concern. Efficacious antimalarial drugs are critical for malaria control and elimination. Continuous monitoring of their efficacy is needed to select the most appropriate treatment for national policies in malaria-endemic countries, and to ensure early detection of, and response to, drug resistance.

Parasite drug resistance results in a delayed or incomplete clearance of parasites from the patient's blood when the person is being treated with an antimalarial. Although therapeutic efficacy studies (TES) remain the gold standard for surveillance of antimalarial drug efficacy, frequent and repeated parasite density counts in malaria cases is logistically demanding and inconvenient for patients and families. Moreover, drug dosage, absorption and metabolism, particularly of artemisinin derivatives (ART), as well as host immunity could influence treatment outcome. Also, while TES are useful to predict the potential occurrence of antimalarial drug resistance, other tools are needed to confirm its presence, such as molecular markers analysis and *in vitro*/*ex vivo* testing.

Protecting the efficacy of artemisinin-based combination therapies (ACTs) as the current first- and second-line treatment for falciparum malaria and for CQ-resistant vivax malaria is among the top global public health priorities. ACTs have been an integral part of the recent success in malaria control worldwide but to date their efficacy is threatened by the onset and spread of resistance to both ART and the partner drugs.

Moreover, although ART resistance alone does not necessarily lead to treatment failure, the reduced efficacy of the ART component places greater demands on the partner drug to clear a larger parasite mass, endangering its future effectiveness. It is also possible for partner drug resistance to emerge independently from ART resistance as demonstrated by the progressive accumulations of mutations in the *pfdhfr/pfdhps* genes of SP resistance observed both in south-eastern India and in south-eastern Nigeria as well as by the fixation of the *pfmdr1* haplotype associated to lumefantrine resistance in south-eastern India reported in this work. Unlike ART resistance, the presence of partner drug resistance brings a high risk of treatment failure in combination therapy and jeopardizes their usage for chemoprophylaxis in vulnerable populations such as pregnant women.

ART and the partner drug play different roles, hence, the efficacy of both drugs must be monitored independently. Monitoring has been central to tracking the evolution of resistance in the Greater Mekong subregions, where multidrug *P. falciparum* resistance is leading to treatment failure with several ACTs. To prevent the occurrence of a similar scenario in the African continent, which today bears the bulk of global falciparum malaria burden, a strict surveillance of molecular markers of drug resistance (such as *K13*) is required. This applies especially to those areas where *in vivo* and *in vitro*/*ex vivo* studies pointed out reduced

ART susceptibility and delayed parasite clearance or where candidate mutations associated with ART resistance have been reported, as observed in the current study in southern Rwanda. Furthermore, the persistent, although slow, westward spread of CQ resistance in *P. vivax* populations from Southeast Asia remains today very worrisome and further investigation on mutations potentially related to such resistance as *pvmdr1* T958M and F1076L are greatly required.

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List of Publications

- **Tacoli C***,* Gai PP, Siegert K, Wedam J, Kulkarni SS, Rasalkar R, Boloor A, Jain A, Mahabala C, Baliga S, Shenoy D, Gai P, Devi R & Mockenhaupt FP (2019) Characterization of *Plasmodium vivax pvmdr1* polymorphisms in isolates from Mangaluru, India. *American Journal of Tropical Medicine & Hygiene* 2019, Jun 17. Doi: 10.4269/ajtmh.19-0224. *[Epub ahead of print]*.

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